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PREFACE

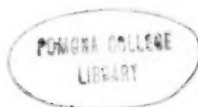In presenting Volume 5 we must record that not all of the articles announced for inclusion in this volume have been received. These circumstances, beyond our control, account for the reduced size of the present book. We expect that Volume 6 will compensate for this shortage. Three of the articles missing this year, Energy Relations in Photosynthesis by D. Burk, The Metabolism of Alkaloids, by K. Möthes, and Tissue Culture by R. J. Gautheret, will be added to the next volume without infringing upon its normal assignment of topics and pages.

We express our gratitude to the authors for the time and care which they have given to the surveys of current literature in divergent areas of plant physiology. We wish also to thank our colleagues who have helped us with advice and suggestions on various aspects of the *Review*. We trust that we may continue to receive tangible expressions of interest in its character and performance.

Some of our colleagues have queried the procedures, common to all *Annual Reviews*, pertaining to the arrangement of literature citations. We admit that this is a debatable matter. The present policy and the reasons for its adoption are stated in the Preface to Volume 20 of the *Annual Review of Biochemistry*.

The rules governing the rotation of the Editorial Committee provide for the retirement of one member each year. To Dr. Paul J. Kramer who is currently retiring, we express our sincere appreciation for his generous and faithful service to the welfare of the *Review*. We welcome Dr. C. A. Swanson as the new member of the Committee.

We wish to acknowledge the devoted help of Beryl Daniel as editorial assistant, and to thank our printers, the George Banta Publishing Company for their unfailing cooperation.

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D.I.A.   L.M.
J.B.   M.W.P.
D.R.G.   C.A.S.
H.B.T.

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TOPICS AND AUTHORS

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CONTENTS

MECHANISM OF ACTION OF MICRONUTRIENT ELEMENTS IN ENZYME SYSTEMS[1,2]

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INTRODUCTION

In recent years the role played by metal-protein complexes in catalytic processes has been given considerable emphasis. A knowledge of the function of metals in enzyme systems is essential for an understanding of their complex effect on the metabolism of both plants and animals. It is clear, however, that merely demonstrating the necessity of a particular metal for an enzyme system does not enable one to describe its physiological effects. This is particularly true when other metals are present. In addition to other factors, one must know the nature of the metal-protein interaction and the influence of various environmental factors on this complex. Is the metal an integral part of the catalytic unit, or is it a dissociable, nonspecific activator functioning by changing the charge distribution on the protein? A complete answer to these and other questions requires a more detailed knowledge of the field than we have at the present time. Rapid progress is being made in this area however; and at the suggestion of the Editors we have restricted our considerations primarily to a review concerned with the mechanism of action of micronutrients in enzyme systems. No attempt will be made to include all those studies in which metals have been shown to influence enzymes, but rather to restrict the discussion to those papers which have a direct bearing on the mechanism of action of the metal. This seems particularly desirable since Hewitt (61) has recently reviewed in some detail the relationships of mineral nutrients to enzyme systems, nutrition and general physiology of plants. Other aspects of mineral nutrition of plants have also been reviewed by Overstreet & Jacobson (120), and Robertson (132).

The task of reviewing the area assigned to the present authors has been made much easier by several recent publications. Lehninger (88), Calvin (23), and Klotz (78) have discussed in some detail the general problems of chelation and the metallo-enzymes chiefly from the standpoint of the physical properties of the ions and the structural linkages between metal ions and organic molecules. Smith (142) has reviewed and presented new data on the function of metals in peptidases. Lardy (86) has discussed the prob-

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[1] The survey of the literature pertaining to this review was concluded in November, 1953.

[2] The following abbreviations will be used: TPN and TPNH, oxidized and reduced triphosphopyridine nucleotide, respectively; DPN and DPNH, oxidized and reduced diphosphopyridine nucleotide, respectively; ATP, ADP, and AMP, adenosine tri-, di-, and monophosphates, respectively; FAD, flavin adenine dinucleotide.

lems of phosphorylation and the influence of metal ions on these processes, and McElroy (94) has reviewed the general problem of multiple metal effects on enzyme systems and on the alteration of enzyme patterns during growth. The present review will of necessity include some of the material which has already been covered in the reviews cited. It is hoped, however, that this necessary duplication will help to clarify the presentation for those readers not directly engaged in this area of research. Although our interest and present discussion will be directed primarily to the micronutrients, we have not hesitated to include other metals such as Mg, K and Na when they have been shown to influence the catalytic properties of a particular protein.

Catalytic Properties of Metallo-Proteins-Mechanism

The basic question concerning the action of micronutrient metals in enzyme-catalyzed reactions is that of their mechanism of action in increasing catalysis after combining with the protein. Many amino acids form metal complexes by coordination through the carboxyl and amine groups. In the case of protein molecules, however, only certain specific polar side chains act as ligands for the formation of metal protein complexes. Klotz (78) has recently reviewed the various properties of polar sidechains of proteins and concludes that the following are known to be involved in complex formation with at least some metals: phosphoric acid, carboxyl, imidazolium, α- and ϵ-ammonium, phenolic, and sulfhydryl groups. Since most proteins usually have at least several of these sidechains, it is to be expected that they will form stable complexes with many metals. From an enzymatic point of view, however, one important function of the metal is to act as a bridge, linking proteins to low molecular weight compounds. Klotz has contributed several excellent examples whereby it is possible to show the binding of uncharged organic molecules to serum albumin provided certain metals are present. Thus, the metal, protein, and organic molecule apparently act together to form a ternary complex. In Klotz's studies on the binding of azopyridine dyes with pepsin, it was possible to show that those metals which were effective in bringing about complex formation (Cu^{++}, Hg^{++}, Ni^{++}, Co^{++}, Zn^{++}, and Mn^{++}) were those which formed chelates with the dye in the absence of protein. Calcium and magnesium failed to combine with the dye and were also inactive in promoting the combination of dye with protein. The studies on the complex formation of metals with proteins and chelate formation of metals with low molecular weight compounds provide the fundamental information necessary for interpreting the mechanism of action of metal ions for certain enzyme catalyzed reactions.

Klotz emphasized three general categories in which metals in combination with proteins or prosthetic groups may act as catalysts: (a) primary effect on the properties of metals, (b) primary effect on the characteristics of the enzyme protein, and (c) cooperative effects of metal and protein. The best known examples of the first group are the enzymes which participate in oxidation-reduction reactions and contain iron or copper as the essential

metal. The catalytic activities of some metalloproteins are observable to a lesser extent in the inorganic form of the metal. As examples, the catalysis of ascorbic acid oxidation by Cu^{++} is greatly enhanced when the copper protein, ascorbic acid oxidase is substituted for inorganic copper; and inorganic iron salts can catalyze the decomposition of H_2O_2 with or without electron donors (peroxidase and catalase activities, respectively). In the case of the iron porphyrin enzymes, it is primarily the porphyrin group which determines the activity of the metal. The copper enzymes recently reviewed by Dawson & Tarpley (34), are discussed in a later section. It is clear from studies made on ascorbic acid oxidase and fatty acid oxidase that the copper, when bound to these specific proteins, acquires characteristics which enable the metal to function efficiently in electron transport. The work of Mahler (97, 98) is particularly significant in this respect for the fatty acid oxidase will, in the absence of copper, result in incomplete electron transport as indicated by dye reduction. The copper is essential for coupling this electron transport system to an unknown "natural" oxidant.

With respect to the second general category, the combination of metals with proteins may lead to an alteration of a number of properties of the latter. The combination will alter the net charge of the protein and from purely electrostatic effects may possibly alter the combination of substrates with enzymes. The recent studies of Sadasivan (134) on acid and alkaline phosphatase activity may be significant in this respect. The results indicate that by changing the ratio of zinc and magnesium one can shift the pH optimum of the enzyme either to an alkaline or acid range. Klotz has emphasized that the titration curves of proteins are affected greatly by combination with metals. If the dissociation of groups essential for enzymatic activity are altered by the presence of metals, it is to be expected that metal activation or depression will vary with pH. Massey (102) has shown that a number of anions such as sulfate, selenate, and borate are effective in activating salt-free crystalline fumarase as well as in shifting the optimum pH to the alkaline side. Likewise Green & Neurath (54) have been able to show an activating effect of such cations as Ca^{++}, Co^{++}, Cd^{++}, and Mn^{++} on the esterase and amidase activities of trypsin even though there is no absolute requirement for a metal. It is not surprising, therefore, to find that metals will alter the pH optimum for enzyme activity. The electrostatic effect of metals on proteins may be responsible for the profound influence of nonessential metals on the metabolism of both plants and animals. In addition to this direct electrostatic effect, however, metals may also activate by the removal of inhibitory substances. One of the more interesting recent examples is furnished by the work of Altmann & Crook (4) on the activation of succinoxidase preparations by chelating agents. The details of these experiments are presented in the next section. Another excellent example on the indirect effects of metals is furnished by Smith (142) on leucine aminopeptidase. The purest enzyme preparations are strongly activated by Mn^{++} while the action of Mg is very poor. However, the stability of the crude or purified enzyme is greatly in-

creased by Mg^{++}. Roche & Lacombe (133) have shown a protecting effect on yeast arginine desiminase (arginine→citrulline+NH_3) by cobalt and nickel in crude extracts but not in partially purified fractions. Metal interaction of the type discussed above is extremely important physiologically when considering the total metal effect as discussed by Arnon (5) and Hewitt (61). This viewpoint receives strong support in the recent observations of Christie, Judah & Rees (26), on the metal requirement of brain mitochondria. Normally brain mitochondria rapidly lose their ability to oxidize various substrates. They found, however, that ATP, DPN, glutathionine, and cobalt added to the mitochondria prolonged the respiratory ability (survival). Copper antagonized the cobalt effect. Treatment of the mitochondria with agents such as phenanthroline greatly reduced the survival time. Cobalt was effective however in preventing the rapid loss of respiratory activity. Fe, Zn, Cd, Mn, and Mg were ineffective. The action of cobalt in maintaining the respiratory activity as well as the ability to esterify inorganic phosphate appears to be particularly significant for brain mitochondria. The recent observations of Ballentine & Stephens (9) that a large amount of inorganic cobalt is incorporated into stable cobalto-protein complexes in the mitochondria of plant tissue may be of some significance in this respect.

With reference to the third general cateogy, the predominant idea in considering the mechanism of action of trace metals has been that metals and proteins may act cooperatively to increase catalytic activity. Two general ideas have been presented to explain a large number of observations. The fact that metals may serve as a bridge between substrate and protein led to the early suggestion that this was the mode of action in such cases as decarboxylations and hydrolysis. Several excellent examples have been presented by Hellerman & Stock (58), Kornberg (80), and Smith (142). In the case of peptidase activity, Smith has emphasized the importance of the formation of a chelate structure between metal ion and the substrate as well as a combination of the metal with the protein as a prerequisite for enzymatic activity. More recently Klotz has emphasized an alternate mechanism for the mode of action of metals in certain types of enzyme catalyzed reactions. He proposes that metals in combination with the protein serve to catalyze the formation of an intermediate which is an essential transition state for the reaction. The details of this mechanism are discussed later. The fundamental difference between this proposal and that discussed by Smith and others is that a chelate structure between metal and substrate is not essential for the formation of the active intermediate. It is clear from data presented in the literature that both hypotheses are applicable. However, differences of opinion exist with regard to particular enzyme systems as indicated in the following examples.

Kornberg, Ochoa & Mehler (82) have emphasized the importance of metal ions in enzymatic decarboxylation reaction, in particular the beta decarboxylation of keto acids. More recently, Steinberger & Westheimer (148) have studied the detailed mechanism of nonenzymatic decarboxylation

of the dimethyl substituted acids and have proposed the formation of a chelate structure between the metal and the carbonyl and alpha carboxyl group of the substrate, as the active intermediate in catalysis. A general scheme for this reaction is presented in Figure 1. The formation of the chelate structure results in an electron shift toward copper and away from the carboxyl, leading to decarboxylation. Williams (168) has emphasized the possible formation of two different types of complexes which would depend upon the pH of the medium. At higher pH's with the loss of two protons, the complexing of the metal with the two carboxyl groups would occur lead-
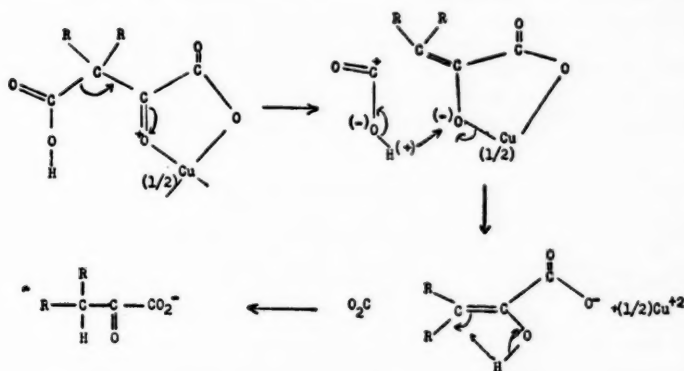
Fig. 1. Metal catalyzed decarboxylation of keto-succinic acid derivatives. [From Calvin (23) after Steinberger & Westheimer (148)].

ing to the formation of an inactive intermediate. The recent work of Albert (2) and of Malmström (100) emphasizes the possibility of formation of several different complexes. In the latter studies two different complexes of manganese with enolase were indicated from kinetic studies. Only one of the complexes was enzymatically active, the other was inhibitory. Thus, in the case of decarboxylation reactions, the decreased enzymatic catalysis at higher pH values may be explained by the formation of inactive chelate structures. A more serious objection may be raised, however, against these proposals when applied to enzymatic reactions. Although zinc, copper, iron, and other metals are effective in catalyzing the nonenzymatic decarboxylation of keto acids these are not the active metals in the enzymatic reactions. Manganese, the most effective metal in enzymatic decarboxylation reactions, is virtually inactive for otherwise catalyzing decarboxylation of keto acids. Vennesland (159) found that Mn^{++} catalysed the oxidative nonenzymatic decarboxylation of oxaloacetic but the product was malonic acid. Recently Kalnitsky (71) demonstrated a similar effect of Mn^{++} on α-ketoglutaric acid oxidation. No CO_2 was evolved under anaerobic conditions and under aerobic conditions

very little succinic acid was formed. The nature of the products are unknown. Apparently Mn^{++} does not catalyse an oxidative decarboxylation similar to that obtained when the enzyme is present. There is no question that the various metals will form bridges between the substrate and the protein but whether these will lead to the formation of an inhibitory complex or an active intermediate must depend upon the reactive groups in the protein and their reaction not only with the metal but with the substrate molecule itself. Additional studies on the nature of the substrate-metal-protein inter- action are necessary before one can accept the formation of a chelate struc- ture as the active intermediate in enzyme catalyzed decarboxylation reac- tions.

Smith (142) has recently reviewed in some detail the characteristics and metal ion requirements of two peptidases: Prolidase, a dipeptidase, which was originally described by Bergmann and Fruton as a catalyst for the hy- drolysis of glycyl-l-proline and leucine aminopeptidase which will hydrolyze
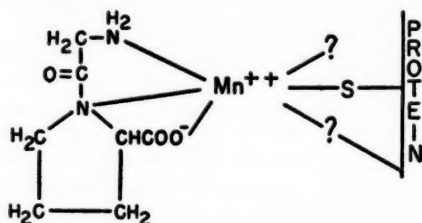
FIG. 2. Postulated coordination of glycyl-L-proline with prolidase.
[From Smith (142).]

many amino acid amides, the usual and most active substrate being L- leucinamide. Prolidase has a specific requirement for Mn^{++} while leucine aminopeptidase can be activated by either Mn^{++} or Mg^{++} although the latter ion is considerably weaker than the former. From a detailed study of the specificity of prolidase as well as a comparison of the metal protein com- plex formation to enzyme activity at various manganese concentrations, Smith has proposed that manganese acts as a ligand in the formation of the active intermediate. The diagram in Figure 2 illustrates this relationship. The main feature of the proposal which is in keeping with the experimental data is that the uncharged amino and the ionized carboxyl groups are the essential points of attachment. Smith proposes therefore that these attach- ments are to the manganese ion. Since such ions tend to form chelates with five-membered rings the third point of attachment as indicated in the dia- gram is suggested. Much of the critical evidence in support of this hypothesis is derived from the action of the enzyme on various substrates. The reason for example, that B-alanyl-L-proline is slowly hydrolysed is due presumably to the fact that a six-membered ring would have to be formed. Klotz has

raised some objections to the proposal of a chelate formation as an essential feature of metal-activated enzymatic hydrolysis. The experimental evidence presented above indicated that Mn^{++} and Mg^{++} are the usual activators of such hydrolytic enzymes, while at the same time they are the poorest chelating metals. In addition, Klotz emphasizes the important point that the products of peptide hydrolysis are usually stronger chelating agents than the initial peptide itself. If a chelate structure were the key intermediate, this would presumably lead to the formation of a stable intermediate, rather than an active one in which the products are actively dissociated from the metal protein. Klotz proposes, therefore, that the primary function of the metal is to favor (or stabilize) the formation of an active intermediate as indicated in the hydrolytic reaction as follows:

$$R\text{-}\underset{O}{\overset{}{C}}\text{-}NHR' + OH^- \longrightarrow R\text{-}\underset{O_-}{\overset{OH}{C}}\text{-}NHR' \longrightarrow R\text{-}C\overset{O^-}{\underset{O}{\diagup}} + H_2NR'$$
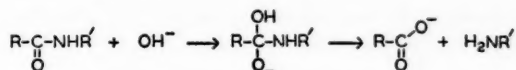
A cationic metal attached to a protein could favor the formation of the intermediate by forming a stable complex as indicated in Figure 3. In addition, the positively charged metal would increase the local concentration of OH^- and these two factors together would tend to speed up the hydrolysis. This proposal suggests, therefore, that the specificity of substrates is due to the interaction of the R and R' groups directly with the protein rather than to their ability to form five-membered chelate structures. Klotz has also extended this idea to a reasonable interpretation of other hydrolytic reactions.
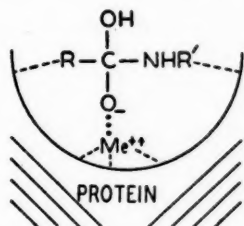
Fig. 3. Stabilization of an intermediate of a reaction by metal protein complex. [From Klotz (78).]

Under appropriate conditions or with suitable substrates such as pyrophosphate containing compounds, Mg^{++} or Mn^{++} may be expected to form chelate structures more readily. Bauer (12) has suggested that the action of inorganic pyrophosphatase depended upon the formation of an enzyme-metal-substrate chelate structure. More recently, Calvin (23) has re-emphasized the importance of the pyrophosphate structure for the linkage of co-enzymes or substrates to the enzyme. The nature of the chelate structure

is shown in Figure 4. Other groups on the protein must be effective in bringing about complex formation with pyrophosphate linkages since metals are not required in all cases. However, in those cases where metals are required, the chelate structure may be an intermediate. The objections raised previously are not applicable in this case since the strength of chelation with Mg^{++} or Mn^{++} would be considerable. In the case of adenosine triphosphatase, inorganic pyrophosphatases, and similar enzymes, the products of hydrolysis would be favored because of the low affinities of inorganic phosphate for Mg^{++} or Mn^{++} as compared to the pyrophosphate. Examples of this type of metal enzyme should be more thoroughly investigated in this respect.

METAL REQUIREMENTS OF ENZYMES

In view of the many enzymes which are activated or dependent upon metal ions, it was deemed best to summarize the more recent findings as
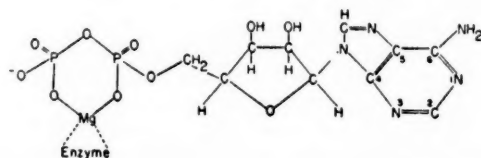
FIG. 4. Binding of pyrophosphate structures (ATP, DPN, TPN, etc.) to enzyme through chelation by Mg^{++}. [After Calvin (23).]

well as the better known examples in tabular form. Omissions have been made but it is hoped that this partial list will serve to bring out the main features of metal ion activation of enzymes.

Carbohydrate metabolism and metal ion catalysis.—It is apparent from Table I that magnesium plays a predominant role in the activity of the various enzymes concerned with carbohydrate metabolism. In most cases Mn^{++} will substitute for magnesium, but the activity is somewhat low. In the case of yeast aldolase Warburg and Christian reported the necessity of Fe^{++}, Zn^{++}, or Co^{++}. The effects and significance of deficiencies of micronutrient elements in the growth medium on aldolase activity are discussed under the section "Metal Deficiencies and Enzyme Constitution."

Hers (59) has recently investigated the relationship of ATP, Mg, Na, and K in the liver fructokinase reaction. By varying the Mg and ATP ratio data are obtained which indicate that the Mg-ATP complex is an actual substrate of the fructokinase reaction. The affinity of the enzyme for the complex was five times greater in the presence of K^+ than in the presence of Na^+. Since high K concentrations were inhibitory and Mg-ATP could competitively reverse this inhibition, Hers suggests that fructokinase has two reactive groups which combine with the metal. The active form of the enzyme is presumably obtained when Mg-ATP complex occupies one site

TABLE I

METAL ACTIVATION OF ENZYMES CONCERNED WITH CARBOHYDRATE METABOLISM

Enzyme	Reaction	Metal*	Reference
Galactokinase	Galactose+ATP→Galactose-1-phosphate+ADP	Mg^{++}	Caputto et al. '50 (25)
Fructokinase	Fructose+ATP→fructose-1-phosphate (or fructose-6-phosphate)+ADP	Mg^{++}, K	Cori & Slein '47 (31) Hers '52 (59)
Glucokinase	Glucose+ATP→glucose 6 phosphate	Mg^{++} Mn^{++}	Cori & Slein '47 (31)
Hexokinase	Fructose ⎫ Glucose ⎬+ATP→hexose-6-phosphate+ADP Mannose ⎭	Mg^{++}, Mn^{++}	Berger et al. '46 (16)
Triokinase	Glyceraldehyde+ATP→3-phosphoglyceraldehyde	Mg^{++}	Hers & Kusaka '53 (60)
Ribokinase	Ribose+ATP→ribose-5-phosphate+ADP	Mg^{++}	Cohen '51 (27)
Gluconokinase	Gluconic acid+ATP→6-phosphogluconic acid+ADP	Mg^{++}	Cohen '51 (27)
Phosphoglucokinase	Glucose-1-phosphate+ATP →glucose-1,6-diphosphate +ADP	Mg^{++}, Mn^{++}	Paladini et al. '49 (121)
Phosphoglucomutase	Glucose-1-P⇌glucose-6-P	Mg^{++}, Mn^{++} Co^{++}, Cr^{++}	Cori, Colowick & Cori '37 (30) Najjar '48 (106) Stickland '49 (150)
Phosphofructokinase	Fructose-6-P+ATP→fructose-1,6-diphosphate+ADP	Mg^{++}	Cori & Slein '47 (31)
Yeast and *Clostridium* Aldolase	Fructose-1,6-P⇌phosphoglyceraldehyde+dioxyacetone phosphate	Fe^{++}, Co^{++} or Zn^{++}	Warburg & Christian '43 (163) Bard & Gunsalus '50 (10)
Phosphoglyceric Acid Kinase	3 phosphoglyceric acid+ATP ⇌1,3 diphosphoglyceric acid	Mg^{++}, Mn^{++}	Bucher '47 (22)
Enolase	2 phosphoglyceric acid⇌1,3 enolphosphopyruvic acid +H_2O	Mg^{++}, Mn^{++} or Zn^{++}	Warburg & Christian '42 (162)
Pyruvic acid kinase	Pyruvic acid+ATP⇌phosphopyruvate+ADP	Mg and K^+, NH_4 or Rb	Kubowitz & Ott '44 (84) Boyer et al. '42 (18)

* The indication of a single metal activator does not mean to imply that this is a specific metal requirement.

and K occupies the other; different combinations are apparently inhibitory. The double metal requirement for maximum enzyme activity lends support to the concept of "metallo-substrates" discussed by Najjar (107). Recently Boyer (19) has studied in detail the metal activation of pyruvic acid kinase from a variety of marine organisms and concluded that K^+ activation is a general phenomenon for this enzyme. Previously, Kachmar & Boyer (69) were able to show that K^+, in addition to Mg^{++} was an absolute requirement for this enzyme from rabbit. In the case of pyruvic acid kinase NH_4^+ and Rb^+ will replace K^+ while Ca^{++} is a potent competitive inhibitor. In many respects the double metal requirement of pyruvic acid kinese is similar to that found for fructokinase. Presumably Mg^{++} would function in combination with ATP while K^+ would combine independently with the enzyme. That Mg^{++} is essential for phosphate-transferring enzymes in general is indicated from the list in Table II. In almost all of the kinases studied Mg^{++} is essential while calcium is usually inhibitory. Some ATPases require Ca^{++} rather than Mg^{++}, but in almost all transfer reactions involving phosphate which have been studied the latter metal is by far the most active. One must conclude with Lardy (86), therefore, that it would be a sound practice to include Mg in reaction mixtures where new ATP requiring reactions are being sought. The possible reason for the unusual participation of Mg^{++} in transfer reactions was discussed in the previous section.

Activation of enzymes in the citric acid cycle.—In addition to its effect on fructokinase and pyruvic acid kinase, K^+ is also known to activate certain enzymes of the citric acid cycle. Stadtman (147) has shown K^+ stimulation of phosphotransacetylase and recently Von Korff (160) has demonstrated a marked stimulation of the enzyme which catalyses the formation of acetyl CoA, pyrophosphate and adenylic acid from acetic acid, ATP, and CoA. Ammonium or Rb ions will also function in this manner. Sodium was a strong inhibitor of this reaction and of some interest is the fact that K^+ will not overcome this inhibition. The results suggest that K may be an absolute requirement for citrate formation from free acetate. Stern, Shapiro & Ochoa (149) have shown already that either Mg^{++} or Mn^{++} is essential for condensing acetyl CoA and oxaloacetic acid to form citrate.

In the presence of the enzyme aconitase, citric acid rapidly equilibrates with *cis*-aconitic acid and isocitric acid. Although aconitase has not been considered as a metal-activated enzyme, recent observations of Dickman & Cloutier (38) are of interest. They found that the loss of aconitase activity in crude heart extracts which have been dialysed or exposed to air could be reversed by ferrous ion. No other metal was effective.

The preparations of isocitric dehydrogenase purified by Grafflin & Ochoa (48) catalyse the conversion of isocitric acid to ketoglutaric acid and requires Mn^{++} for maximum activity. Ochoa and co-workers (82) have suggested that Mn^{++} is essential primarily for the enzymatic decarboxylation of oxalosuccinic rather than the dehydrogenation reaction. This is in keeping with the suggestion of Kornberg, Mehler & Ochoa (81) for oxaloacetic acid decarboxylation. Recently, however, Lotspeich & Peters (92) demonstrated

TABLE II

METAL ACTIVATION IN OTHER PHOSPHORYLATION REACTIONS

Enzyme	Reaction	Metal*	Reference
Myokinase (adenylate kinase)	$2 \text{ ADP} \rightleftharpoons \text{AMP} + \text{ATP}$	Mg^{++}	Colowick & Kalckar '43 (28)
Adenosine kinase	Adenosine $+\text{ATP} \rightarrow \text{ADP} +\text{AMP}$	$Mg,^{++} Mn^{++}$	Caputto et al. 50 (25)
Creatine kinase	Creatine $+\text{ATP} \rightleftharpoons$ Creatine Phos. $+\text{ADP}$	Mg^{++}	Lehmann '35 (87)
Arginine kinase	Arginine $+\text{ATP} \rightleftharpoons$ Arginine Phos. $+\text{ADP}$	Ca^{++}, Mn^{++} Mg^{++}	Szorenyii et al. '49 (155)
Flavokinase	Riboflavin $+\text{ATP} \rightarrow$ riboflavin Phos. $+\text{ADP}$	Mg^{++}	Kearney & England '51 (72)
DPN kinase	$\text{DPN} + \text{ATP} \rightarrow \text{TPN} + \text{ADP}$	Mg^{++}, Mn^{++}	Kornberg '50 (80)
Firefly luciferase	$\text{ATP} + \text{LH}_2 + \text{O}_2 \rightarrow \text{LIGHT} +\text{L}$	Mg^{++}, Mn^{++}	McElroy & Strehler '49 (95)
ATPase	$\text{ATP} + \text{H}_2\text{O} \rightarrow \text{AMP} + 2\text{PO}_4$	Mg^{++} or Ca^{++}	Numerous
Yeast apyrase	" " " "	Mn^{++}	Meyerhof '45 (104)
Potato apyrase	" " " "	Ca^{++}	Kalckar '44 (70)
Nicotinamide Methyl kinase	Methylation of Nicotinamide by Methionine	Mg^{++}	Cantoni '50 (24)
Glytamyl transferase	Glutamine $+\text{NH}_2\text{OH} +\text{ATP} \rightarrow$ glutamohydroxamic acid $+\text{NH}_3$	Mn^{++}	Stumpf & Loomis '50 (153)
Glutamine Synthesizing Enzyme	Glutamic $+\text{NH}_3 + \text{ATP} \rightarrow$ glutamine $+\text{ADP}$	Mg^{++} Mg^{++}, Mn^{++}	Speck '47 (144) Elliott '51 (41)
Dephospho CoA kinase	Dephospho CoA $+\text{ATP} \rightarrow$ CoA $+\text{ADP}$	Mg^{++}, Mn^{++}	Wang & Kaplan '53 (161)

* The indication of a single metal activator does not mean to imply that this is a specific metal requirement.

appreciable quantities of Mn^{++} in the enzyme preparation and suggest that Mn^{++} is actually essential for dehydrogenase activity. They found that highly diluted enzyme was inactive unless Mn^{++} was added and that the inhibitory effects of high phosphate concentration could be overcome by this metal. The evidence is not conclusive, however, since the enzymatic removal of oxalosuccinic acid, presumably a Mn-requiring reaction, may be affecting the preceeding dehydrogenase reaction by a mass action effect. The rapid removal of oxalosuccinic acid by other systems in the absence of Mn^{++} would be desirable. Hartman & Kalnitsky (57) have shown that Mg^{++} had no effect on the oxidation of citrate when added to kidney cortex mitochondria after the addition of Mn.$^{++}$ However, if Mg^{++} is added before Mn^{++}, there was a marked inhibition which was shown to be competitive. More recently these workers (56) have observed the same relationship with copper and manganese.

Although several different preparations of succinic dehydrogenase and succinoxidase have been described, the general reactions and metal requirements are still not clearly defined. Keilin & Hartree (73) suggested that the previous observations on the stimulation of succinoxidase by Ca^{++} and Al^{+++} were due to the formation of precipitates of these metals which acted as surfaces for the favorable orientation of the enzyme. Recently, however, Altmann & Crook (4) have presented convincing evidence that the effectiveness of the metal gels is due to their ability to remove inhibitory metals. Versene, 8-hydroxyquinoline, and pyrophosphate as well as purification of succinate to remove heavy metals, stimulate the enzyme in bicarbonate buffer. The earlier observation that high phosphate concentrations will give maximum activity of the enzyme can also be explained on this basis. Other metal requiring enzymes of the citric acid cycle are included in Table III.

TABLE III

THE ROLE OF METALS IN THE ACTIVATION OF ENZYMES OF THE CITRIC ACID CYCLE

Enzyme	Reaction	Metal*	Reference
Pyruvic carboxylase	Pyruvic acid→Acetaldehyde+CO_2	Mg^{++}, Mn^{++}	Auhagen '32 (6) Green et al. '41 (52)
Pyruvic oxidase	Pyruvic acid+H_3PO_4 →Acetyl-P+CO_2	Mg^{++}, Mn^{++}	Lipmann '44 (91)
Pyruvic oxidase	Pyruvic acid+CoA →Acetyl-CoA+CO_2	Mg^{++}	Korkes '51 (79) Schweet et al. '51 (140)
Oxaloacetic decarboxylase	Oxaloacetic acid→Pyruvic acid+CO_2	Mg^{++}, Co^{++} Zn^{++}, Mn^{++}	Speck '48 (145) Plaut & Lardy '49 (125)
Transacetylase	Acetylphosphate+CoA ⇄PO_4+Acetyl CoA	Mg^{++}, K^+	Stadtman '50 (146) Stadtman '52 (147)
Isocitric dehydrogenase	Isocitric Acid+TPN ⇄oxalosuccinic+TPNH	Mg^{++}, Mn^{++}	Adler et al. '39 (1)
Oxalosuccinic decarboxylase	Oxalosuccinic→ketoglutaric acid+CO_2+O_2	Mn^{++}	Kornberg et al. '48 (82)
a-ketoglutarate oxidase (plant)	a-ketoglutaric acid→succinic acid+CO_2	Mg^{++}, Mn^{++}	Schales et al. '50 (139)
Succinic dehydrogenase	Succinic acid→fumaric acid+2H	Ca^{++} Al^{+++}, Cr^{++}	Axelrod et al. '42 (7) Horecker et al. '39 (64)
"Malic enzyme"	Malate+TPN→Pyruvate +CO_2+TPNH	Mn^{++} Co^{++}	Salles & Ochoa '50 (135) Conn et al. '49 (29)
Condensing enzyme	Oxaloacetic acid+Acetyl CoA⇄citric acid	Mg^{++}, Mn^{++}	Stern et al. '50 (149)

* The indication of a single metal activator does not mean to imply that this is a specific metal requirement.

Whereas Mg^{++} plays a predominate role in the glycolytic cycle in which phosphate transfer is essential, it appears that Mn^{++} is the predominate ion in the citric acid cycle. This effect of Mn^{++} is related to the important oxidative and nonoxidative decarboxylation steps which occur during the metabolism of di- and tricarboxylic acids.

Multiple metal activation of enzymes.—It is clear from the previous tables that certain enzymes may be activated by a number of metals. The enzymes listed in Table IV are some additional examples in which a variety of metals

TABLE IV

MULTIPLE METAL ACTIVATION OF ENZYMES

Enzyme	Reaction	Metal	Reference
Yeast phosphatase	Glycerophosphate →glycerol+PO_4	Mg^{++}, Mn^{++}, Co^{++} Fe^{++}, Ni^{++}	Massart & Van-dendriessche '40 (101)
Acid and alkaline phosphatases	Numerous substrates	Mg^{++}, Mn^{++} and others	See Tauber '49 (156)
Arginase	Arginine+H_2O ornithine+urea	Mn^{++}, Co^{++}, Ni and Fe	Stock, Perkins, & Hellerman '38 (151)
Lecithinase	Lecithin→phosphoryl-choline+diglyceride	Ca^{++}, Mg^{++}, Co^{++} Zn^{++}, Mn^{++}	Zamecnik, *et al.* '47 (174)
Cysteine desulfhydrase	Cysteine→H_2S+NH_3 +$CH_3COCOOH$	Zn^{++}, Mg^{++}, Mn^{++}	Binkley '43 (17)
Pectinpoly-galacturonase	Hydrolysis of pectic polyuronides	Ca^{++}, Na^+, Al^{+++}	Pallman et al. '46 (122)
Desoxyribonuclease	Depolymerization	Mg^{++}, Mn^{++} Co^{++}, Fe^{++}	Miyaji & Green-stein '51 (105)
Histidine deaminase	Histidine→uroconic acid+NH_3	Zn^{++}, Hg^{++}, Cd^{++}	Suda *et al.* '53 (154)

will serve as activators. Arnon (5) and Hewitt (61) have recently discussed this general problem of multiple activation with respect to essentiality of micronutrients in plant growth. They have emphasized the difficulty of considering criteria of essentiality with respect to micronutrients in enzyme systems where multiple activation is possible. The demonstration that certain enzymes can be activated by nonessential metals, such as nickel, indicates quite clearly that the total metal environment, including essential and nonessential metals, will have a profound influence on metabolic patterns. It seems clear, however, that it is only necessary to show one vital and irreplacable function of a metal to establish its essentiality [cf. (5)]. As discussed in the next section, this has been demonstrated in a reasonably conclusive manner for all the presently considered essential micronutrients except boron.

Specific metal components of enzymes.—The enzymes in Table V have a

TABLE V

SPECIFIC METAL ACTIVATION OF VARIOUS ENZYMES

Enzyme	Reaction	Metal	Reference
Carbonic anhydrase	$CO_2H_2O \rightleftharpoons H_2CO_3$	Zn	Keilin & Mann '40 (74)
Dehydropeptidase	Glycyldehydrophenylanine $\rightarrow$glycine$+NH_3+$phenyl-pyruvic A	Zn	Yudkin & Fruton '47 (173)
Glycylglycine dipeptidase	Glycylglycine$\rightarrow$glycine	Zn	Linderstrøm-Lang '34 (90)
Inorganic pyrophosphatase	Pyrophosphate$+H_2O\rightarrow PO_4$	Mg	Bailey & Webb '44 (8)
Carboxypeptidase	Chloroacetyl-tyrosine$\rightarrow$tyrosine	Mg	Smith & Hanson '49 (143)
Fumaric hydrogenase	Fumaric Acid$+2H\rightarrow$Succinic Acid	Fe	Harrison '53 (55)
Catalase	$2H_2O_2\rightarrow 2H_2O+O_2$	Fe	Granick & Gilder '47 (50)
			Lemberg & Legge '47 (89)
Peroxidase	H_2O_2 oxidation of aromatic amines and other compounds	Fe	Lemberg & Legge '47 (89)
Cytochromes	Electron transport	Fe	Lemberg & Legge '47 (89)
DPNH-cytochrome-c reductase	DPNH$+$cytochrome-c $(Fe^{+++})\rightarrow$DPN $+$cyto-chrome-c (Fe^{++})	Fe	Mahler & Elowe '53 (99)
Tyrosinase	Tyrosine$+\frac{1}{2}O_2\rightarrow$hallochrome	Cu	Nelson & Dawson '44 (116)
Laccase	Phenols$\rightarrow$ortho and para-quinones	Cu	Dawson '50 (33)
Ascorbic acid oxidase	Ascorbic acid$\rightarrow$dehydroascorbic A	Cu	Dawson '50 (33)
Butyryl CoA dehydrogenase	Butyryl CoA-2e$\rightarrow$crotonyl-CoA	Cu	Mahler '53 (97)
Prolidase	Glycylproline$\rightarrow$proline	Mn	Fruton '46 (47)
Nitrate reductase	$NO_3^-+TPNH+H^+\rightarrow NO_2^-$ $+TPN^++H_2O$	Mo	Nicholas, Nason & McElroy '53 (117–19)
Xanthine oxidase	Xanthine$+O_2\rightarrow H_2O+$uric acid	Mo	De Renzo et al. '53 (35, 36, 37)
			Green & Beinert '53 (51)
			Richert & Westerfield '53 (131)
			Totter et al. '53 (157)

specific requirement for the metal listed. Since most of these enzymes have been discussed in some detail in a number of reviews, (34, 49, 50) we have restricted the present summary to very recent developments.

METALLO-FLAVOPROTEINS

During the past year the involvement of metals in flavoprotein catalyses has been demonstrated simultaneously and independently for five different enzymes.

Butyryl CoA dehydrogenase.—Mahler (97, 98) has recently demonstrated that FAD and ionic copper are integral parts of the prosthetic group of one of the enzymes concerned in fatty acid oxidation, butyryl CoA dehydrogenase (53). The purified enzyme obtained from liver is colored a deep, brilliant green, presumably due to a copper complexing reaction, and catalyses the oxidation of butyryl CoA to crotonyl CoA. It was shown that copper is the significant metal bound to the protein, that it occurs in the ratio of 2 M of Cu^{++}/FAD, and that the enzyme has three major absorption peaks (max. 355, 432.5, and 685 mμ). Upon treatment with butyryl CoA the three absorption peaks decline as a result of reduction. Prolonged dialysis of the enzyme against cyanide causes the peak at 685 mμ, which is related to the presence of copper on the enzyme, to disappear and the green color of the enzyme to change to yellow. The flavin peaks (355 and 432.5 mμ) of the cyanide-treated enzyme, upon reduction by substrate, is similar to that of reduced untreated enzyme. The results indicate that copper does not play a role in the primary reduction of the enzyme-bound flavin. In the absence of copper if ferricytochrome-*c* or ferricyanide are used as terminal electron acceptors, the activity of the enzyme is greatly reduced, full activity being restored only after preincubation of the enzyme with Cu^{++}. The oxidation-reduction reactions mediated by the enzyme have been represented as follows:

$$\underbrace{\text{Butyryl CoA} \xrightarrow{2e^-} \text{FAD} \xrightarrow{e^-} Cu^{++} \to (Fe^{+++})}_{\text{Enzyme}}$$
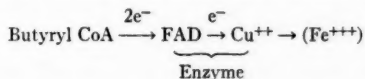
The exact mechanism of the copper-mediated portion of the reaction, namely the normal physiological electron acceptor, is still obscure. It is not oxygen, since no uptake of gas occurs; nor can it be cytochrome-*c*, since reduction of this acceptor is "almost certainly too slow to be of physiological importance." It is suggested that the "natural" oxidant, which will be capable of undergoing one-electron changes, is perhaps a ferri-haematoprotein.

Nitrate reductase.—The isolation and characterization of nitrate reductase from *Neurospora* and soybean leaves, by Nason & Evans (42, 108) has clarified the hitherto obscure mechanism by which nitrate is converted to nitrite in various plants. The enzyme has been purified approximately 70-fold from both sources and has been shown to be a flavoprotein. By reactivation studies, fluorometric analysis, and the D-amino acid oxidase test the prosthetic group has been identified as flavin adenine dinucleotide. Inhibi-

tion by p-chloromercuribenzoate which can be reversed by cysteine or glutathione indicates the necessity of a sulfhydryl group for catalytic action. The enzyme from *Neurospora* catalyses the reduction of nitrate to nitrite according to the equation:
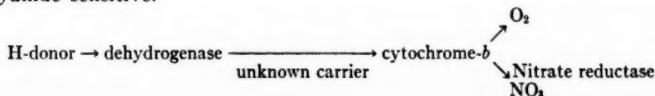
$$NO_3^- + TPNH + H^+ \rightarrow NO_2^- + TPN^+ + H_2O$$

and is relatively specific for TPNH as the electron donor. In the case of the enzyme from soybean leaves TPNH and DPNH are equally effective. By the use of grana and purified enzyme from soybean leaves the photochemical reduction of nitrate was demonstrated, thus pointing to a possible mechanism for the role of light in the reduction of nitrate by higher plants. Nitrate reductase, which is adaptive to nitrate or nitrite, but not to ammonia or alanine, is present in other higher plants and fungi. The metallo nature of the enzyme was indicated by its sensitivity to metal binding agents such as cyanide, azide, thiourea, potassium ethyl xanthate, o-phenantholine, and 8-hydroxyquinoline. Nicholas, Nason & McElroy (117, 118, 119) have identified the metal constituent as molybdenum. An examination of nitrate reductase activity in cell free extracts of mycelia individually deficient in each of the micronutrient elements showed that only a molybdenum deficiency caused a decrease in specific activity of the enzyme. The enzyme could be completely inactivated by dialysis against cyanide. Following subsequent dialysis against phosphate buffer and glutathione to remove excess cyanide, reactivation of the enzyme was obtained only with molybdenum (as molybdenum trioxide or sodium molybdate). In addition to these studies it was shown that the molybdenum content parallels specific activity during enzyme fractionation. Although the mechanism of action of molybdenum is not known it is possible that the metal may be acting in an anionic form as a carrier which shuttles electrons from reduced FAD to nitrate to form nitrite as follows:

$$TPNH \rightarrow FAD \rightarrow Mo \rightarrow NO_3^-$$
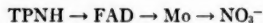
Nitrate reductase from higher plants appears to be quite different from that observed in *Escherichia coli*. However, the nature and identity of the components of the enzyme from *E. coli* still remain obscure, and in most of the enzymatic studies reduced dyes have been used as the electron donor (39, 40, 67, 136). The suggestion of Sato & Egami (136) that nitrate reductase of *E. coli* is an iron enzyme, very likely identical with cytochrome-b, has recently been retracted by Sato & Niwa (137). Nevertheless, they believed that cytochrome-b is closely associated with the nitrate reductase mechanism of *E. coli* and propose the following scheme to account for the observation that cytochrome-b is cyanide insensitive (123) while nitrate reductase is cyanide sensitive.

H-donor → dehydrogenase ⎯⎯⎯⎯⎯⎯⎯⎯→ cytochrome-b ⟶ O_2
                            unknown carrier   ↘ Nitrate reductase
                                                        NO_3

Sato & Niwa (137) claim that iron is the metal component of nitrate reductase from *E. coli* based on carbon monoxide inhibition of the enzyme and its reversal by light. In addition, they cite unpublished data that *E. coli* grown on iron-deficient media has a considerably lowered activity as compared to cells supplied with a sufficient amount of iron. In view of the controversial ideas and observations concerning nitrate reductase of *E. coli* the status of this enzyme with reference to mechanism and mode of action is still unsettled.

Xanthine oxidase.—The involvement of molybdenum in the activity of xanthine oxidase, a flavoprotein of liver, intestine, and milk has been indicated by a number of workers. It has been known for some time that a factor besides riboflavin and protein was present in certain foods which raised the xanthine oxidase activity of liver and intestine. Richert & Westerfield (131) and De Renzo and co-workers (35, 36, 37) independently identified the dietary factor ("Xanthine Oxidase factor") in natural materials as molybdenum. That molybdenum is a component of xanthine oxidase has not been conclusively established. These workers report that it has been impossible to stimulate, by added molybdate, the xanthine oxidase activity of intestinal homogenates with low or high initial activities. Similar observations have been made with dialyzed enzymes and preparations from molybdenum-deficient rats. This would be indicative of the absence or impairment of the apoenzyme in molybdenum deficiency if one assumes that molybdenum is a component of the active enzyme. However, the possibility has not been eliminated that molybdenum exerts its influence on xanthine oxidase activity by indirectly affecting the formation of the protein moiety of the enzyme. Evidence in favor of the action of molybdenum as a constituent of the enzyme is suggested by the observation that purified preparations of milk xanthine oxidase contain the "xanthine oxidase factor" or molybdenum (130). In further support of this idea is the work of Green & Beinert (51) and Totter *et al.* (157) who have isolated xanthine oxidase from cream and analyzed preparations at different stages of purity. Their results showed molybdenum to be present in the ratio of one atom of metal per 2 molecules of flavin. Totter *et al.* have also reported that the molybdenum found in xanthine oxidase is non-exchangeable and is not removed by dialysis against water. Dialysis against cyanide followed by dialysis against water or buffer would be a more critical experiment. The data, however, still do not exclude the possibility that a molybdate-protein complex, unrelated to xanthine oxidase activity, has accompanied xanthine oxidase activity during purification. In the opinion of the reviewers further evidence is still necessary to implicate molybdenum conclusively as a constituent of the enzyme. The loss of enzyme activity upon removal of molybdenum, and its reactivation, if possible, upon restoration of the specific metal, together with the failure of other metals to accompany xanthine oxidase activity consistently are necessary as final evidence. If molybdenum is a constituent of xanthine oxidase, as it tentatively appears to be, it is very likely functioning in a manner similar to that proposed for

molybdenum in nitrate reductase and for Cu in the butyryl CoA dehydrogenase.

It is clear that molybdenum is required, although at decreased levels, when ammonia serves as the sole nitrogen source for *Neurospora* and *Aspergillus* indicating an involvement in metabolic processes other than nitrate reduction. In this connection the reduced glutamic dehydrogenase activity in molybdenum deficient tissues of *Neurospora* (117) may be of interest. With reference to other possible functions of molybdenum, Hewitt & Agarwala (63) report that a molybdenum deficiency in representative higher plants greatly depresses the reduction of triphenyltetrazolium by the tissues. This is in line with the results previously reported by Evans *et al.* (43) that homogenates of molybdenum-deficient lucerne foliage had a decreased hydrogenase activity as measured by methylene blue reduction. The significance of metal deficiency effect on enzyme activity such as reported above, is discussed in the section, "Metal Deficiencies and Enzyme Constitution." Hewitt *et al.* (62) reported a marked reduction in ascorbic acid content in molybdenum-deficient plants. Upon injection of molybdate, the ascorbic acid level was increased considerably within 24 hr. They relate these changes in ascorbic acid content with nitrate reduction. The interesting catalytic effect of molybdate on acid hydrolysis of phosphocreatine described by Fiske & Subbarow (45) has been further investigated. Barker, Ennor & Harcourt (11) showed the products to be orthophosphate, creatine and creatinine, the relative proportions of creatine and creatinine being a function of molybdate concentration. At 4×10^{-4} M molybdate only 10 per cent was converted to creatine. Attempts to find an enzyme system or an activating effect of molybdenum in the possible enzymatic transformation of phosphocreatine to creatinine using dialyzed homogenates of skeletal muscle, liver, and kidney were unsuccessful. Weil-Mahlherbe & Green (164) suggest that the catalytic effect of molybdate on the hydrolysis of particular organic phosphate bonds is due to the formation of a complex with substrate as the first step in the breaking of the phosphate bond. Complexes of molybdate with organic compounds, phosphorylated or not, are well known, and these may explain the inhibition of acid phosphatase by molybdate.

Fumaric hydrogenase.—Harrison (55) has reported that the yeast FAD-enzyme described by Fischer & Eysenbach (44) which catalyses the reduction of fumarate to succinate by leucodyes is restored, after dialysis, to full activity by the addition of ferrous ions. Other metals such as Mn^{++} are ineffective. This enzyme may be related in function to Keilin-Hartree preparations of succinic dehydrogenase from heart muscle which also loses activity on dialysis. Fe^{++} is also effective in restoring activity in these preparations. This would appear to constitute another metallo-flavoprotein in which Fe^{++} is required for electron transfer in succinate-fumarate systems.

DPNH-Cytochrome-c reductase.—Mahler & Elowe (99) have made the interesting observation that iron is necessary for the flavin enzyme which reduces cytochrome-*c* using DPNH as the electron donor. In the absence of iron the reduction of cytochrome-*c* is greatly inhibited, while the diaphorase

activity functions normally. In other words, like copper in the butyryl CoA dehydrogenase, Fe is essential for coupling electron transfer from the flavin component to cytochrome. Mahler and Elowe suggest therefore that diaphorase, the flavoprotein which catalyses the reduction of dyes but not cytochrome-c by DPNH, may be a transformed cytochrome-c reductase. This coupling of pyridine nucleotide-flavin reducing system to the cytochrome system is similar in many respects, to the coupling of the succinic dehydrogenase to cytochrome-c by the Slater factor (141a). Slater has suggested that in the latter system a protohaematin is necessary for the coupling between cytochrome-b and cytochrome-c.

It is of interest that such dissimilar reactions as nitrate reduction, fatty acid oxidation, purine oxidation, and dicarboxylic acid reduction are catalysed by such similar enzymes. On the other hand, the property common to all these reactions is that they are involved in electron transport systems. The fact that Cu, Fe, and Mo are the metal components involved emphasized the important role these metals play in electron transport.

The Copper Enzymes

One of the general properties ascribed to the copper-containing enzymes by Dawson & Tarpley (34) has been the catalysis of the direct oxidation of their respective substrates by atmospheric oxygen according to the equation:

$$BH_2 + \tfrac{1}{2}O_2 \xrightarrow{\text{Cu-Enzyme}} B + H_2O$$
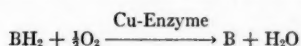
and their failure to function anaerobically. The characterization of the new copper enzyme, butyryl CoA dehydrogenase, by Mahler (97) (already discussed under the section dealing with metallo-flavoproteins), in which the oxidation-reduction reaction appears to involve a reversible $Cu^{++} - Cu^+$ cycle exclusive of oxygen, invalidates the above generalization as characteristic of the copper enzymes.

In addition to the demonstration of copper as part of the prosthetic group of butyryl CoA dehydrogenase, there recently have been a number of interesting findings pertaining to the mechanism of action of other copper-protein systems. The probable mechanism of a reversible $Cu^{++} - Cu^+$ cycle already experimentally indicated by Kubowitz (83) for the action of polyphenol oxidase from potato, is also strongly suggested by the experimental results of Dawson and co-workers (33, 68) for ascorbic acid oxidase. Using radioactive Cu^{64} as the tracer these workers were unable to show an exchange reaction between the copper of ascorbic acid oxidase with ionic copper in the resting enzyme or in the enzyme which has been inactivated during the course of the reaction. These observations support the view that the copper-protein bond in the resting enzyme is of a nondissociable nature. This is contrary to the view of Lampitt & Clayson (85) who have suggested that the catalytic activity of ascorbic acid oxidase may be attributed to traces of ionic copper resulting from an ionization of copper bound to nonspecific protein matter or other colloidal material. However, a significant amount of exchange was
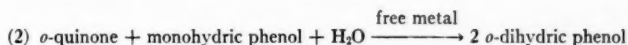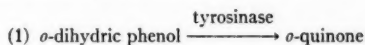
observed when the enzyme was actively catalyzing the oxidation of ascorbic acid in an aerobic system. No exchange occurred in the absence of oxygen even with the substrate present. These observations have been interpreted to indicate that the copper of ascorbic acid oxidase initially in the divalent state, shuttles reversibly between the divalent and monovalent states during enzyme catalysis. The exchange occurs only when the copper of the enzyme appears in the monovalent state during this oxidation-reduction cycle. The presence of both ascorbic acid and oxygen are apparently necessary for the $Cu^{++}-Cu^{+}$ reaction of the enzyme to occur. The results suggest the formation of a ternary complex involving oxygen, ascorbic acid, and enzyme as a necessary intermediate. The assumption that the exchange is limited to monovalent forms of copper is supported from observations concerning the structure and bond strengths of both di- and mono-covalent complexes of copper. The usual type of covalent complex formed by divalent copper is one with a coordination number of 4, having a square coplanar configuration for the directed valences of the copper atom (167). Mono-covalent copper with a coordination number of 4 exists, however, in a tetrahedral configuration. Since square coplanar bonds are considered to be much stronger than the tetrahedral bonds (124), it is likely that the exchange would be favored when the copper bond is changed from the coplanar to a tetrahedral configuration during reduction. The report (66) that covalent nickel compounds of the square planar type do not exchange with radioactive nickelous ions in solution, whereas the tetrahedral nickel complexes which are of a weaker bond strength do exchange, tends to support the above hypothesis. It is of interest that there was no exchange of radioactive copper with that of the reaction inactivated enzyme, indicating that the inactivation is not due to an irreversible loss of copper from the enzyme.

A specific respiratory function for ascorbic acid as part of a DPNH-oxidizing system has been proposed by James *et al.* (65) based on experiments using expressed juice of barley seedlings. Subsequently, Mathews (103) observed the enzymatic oxidation of DPNH by a protein fraction from peas in the presence of ascorbic acid and oxygen. Beevers (14) has made similar observations with a cucumber extract containing ascorbic acid oxidase, while Kern & Racker (75) have shown DPHN oxidation by a protein fraction from yeast upon the addition of ascorbic acid and ascorbic acid oxidase. Dehydroascorbic acid failed to serve as an oxidant of DPNH in the above systems (75). Nason *et al.* (114) have shown that the oxidation of ascorbic acid by ascorbic acid oxidase, Cu^{++} or Fe^{+++} gives rise to a labile intermediate between ascorbic and dehydroascorbic acids which serves specifically as an electron acceptor for the catalysed oxidation of DPNH or TPNH by an enzyme fraction from peas. The presence of such an intermediate in ascorbic acid oxidase action provides a direct link between respiratory substrates and oxygen by way of pyridine nucleotides. While the nature of the above intermediate remains to be clarified, one of the possibilities is a free radical such as monodehydroascorbic acid. A semiquinone radical has been pictured as an

intermediate by Weissberger *et al.*, (165, 166) in the mechanism of copper ion catalysis of ascorbic acid oxidation. A coordination complex between Cu^{++} and monovalent ascorbic ion is envisaged as undergoing an intra-molecular electron shift whereby Cu^{++} is reduced to Cu^+ at the expense of ascorbate ion which becomes a free radical. The Cu^+ is released from the co-ordination complex and oxidized to Cu^{++} by oxygen, while the free radical of the ascorbate ion is further oxidized to dehydroascorbic acid.

The characterization by Wosilait *et al.* (171, 172) of the enzyme from peas which catalyses the reduction of various quinones by reduced pyridine nucleotides has helped clarify the possible pathways of electron transfer by way of the phenolases. In the presence of the copper enzymes, laccase, and tyrosinase it is possible to couple the quinone reductase system to molecular oxygen as the ultimate electron acceptor. The results imply a broader role for these copper oxidases in the metabolism of higher plants, i.e., the copper enzyme systems by way of quinone reductase and pyridine nucleotides may act as terminal oxidases for respiratory substrates, a viewpoint which has been emphasized by many workers [see Dawson & Tarpley (34); Nelson (115)].

Kertesz (76) using purified potato enzyme studied the mechanism of tyro-sinase oxidation of monohydric phenols. He observed that additions of Cu^{++} determined further increases of tyrosinase activity on tyrosine; and that Co, Va, and Ni could replace Cu^{++}, though less effectively. He has interpreted these data to mean that monophenolase activity of tyrosinase belongs to free metallic ions which hasten the nonenzymatic reaction between *o*-quinones and monohydric phenols as follows:

(1) *o*-dihydric phenol $\xrightarrow{\text{tyrosinase}}$ *o*-quinone

(2) *o*-quinone + monohydric phenol + H_2O $\xrightarrow{\text{free metal}}$ 2 *o*-dihydric phenol

Thus tyrosinase is identified as a complex system composed of an *o*-dihydric phenol (or *o*-quinone), an enzyme specific for *o*-dihydric phenols, and of free metallic ions, the latter accelerating the spontaneous reaction between *o*-quinone and tyrosine. This is contrary to the views of Dawson and co-workers (34) who ascribe monophenolase activity to the tyrosinase molecule.

METAL DEFICIENCIES AND ENZYME CONSTITUTION

The necessity of a metal ion for the activity or synthesis of specific enzymes may be revealed by growing the organism under conditions of metal deficiency and comparing its enzyme systems with those of normal tissues. Reed (128) in earlier work on zinc deficiency in tomatoes, reported that the dehydrogenase activity was decreased, while the quinones arising by action of phenol oxidases were not reduced. In addition, the accumulation of in-organic phosphate suggested a possible role of zinc in the activation of a phosphate transferring enzyme, possible hexokinase. Waring & Werkman

(170) reported that iron deficiency in *Aerobacter indolgenes* resulted in the suppression of a number of enzymes; while Foster & Denison (46) found pyruvic carboxylase to be absent in extracts of zinc-deficient *Rhizopus nigricans*. Zinc is not a constituent of the pyruvic carboxylase, but is necessary for the synthesis of the enzyme itself. It has also been shown that the activity of an enzyme concerned in the synthesis of tryptophane (indole+serine →tryptophan) is almost completely suppressed in cell-free extracts of zinc-deficient Neurospora (109). An extension of the latter finding to the tomato plant might account for the observations that zinc is required directly for the synthesis of tryptophan and indirectly for the synthesis of auxins (141, 158).

More extensive studies of effects of metal deficiencies on various enzymes have brought to light some interesting aspects of micronutrient element function. A deficiency of zinc or biotin in *Neurospora* does not simply lead to the production of less mycellium but actually results in the production of mycelia having drastically altered metabolic characteristics as indicated by the marked changes in enzymatic constitution (109). The alterations involve not only the virtual disappearance of certain enzyme activities such as that of alcohol dehydrogenase and of the enzyme which condenses indole and serine to form tryptophan but also remarkable increases in the activity of other enzymes. An example of the latter is the 10- to 20-fold rise in the concentration of diphosphopyridine nucleotidase (DNPase) an enzyme which splits DPN at the nicotinamide riboside linkage. On the other hand, certain enzymes including fumarase, hexokinase, aldolase, and triosephosphate dehydrogenase were unaffected. Apparently zinc deficiency leads to an actual decrease in the concentration of other enzyme proteins. Determinations of total protein in the extracts show, however, that the net effect is a profound decrease in protein concentration. Since this is not changed by supplementing the zinc-deficient medium with a full complement of known amino acids, purines, pyrimidines, and vitamins it would seem that the basic defect in zinc deficiency lies not in the synthesis of these simple units, but in their subsequent metabolism, certainly in the failure to synthesize enzyme proteins in the normal fashion. A working hypothesis to explain the above phenomena is that those enzymes which increase in nutritionally-deficient cells are proteins of relatively simple structure, the synthesis of which can proceed even in the absence of certain key reactions which are necessary for the building of more complex protein molecules. A metal deficiency may eliminate one of several competing reactions for available amino nitrogen resulting, therefore, in a relative increase in certain enzymes and decreases in others. Supporting the latter concept is the fact that protein synthesized in the zinc-deficient mycelia is not available for new enzyme synthesis. Nitrogen, as well as zinc, is essential for the restoration of the DPNase and alcohol dehydrogenase levels of zinc-deficient mats (110). The results suggest that new synthesis of protein from amino nitrogen must occur for the restoration of the normal enzyme pattern. It may be that certain enzyme proteins

are stabilized by the presence of specific metals. Examples of this effect have been discussed in a previous section.

A somewhat comparable study with individual deficiencies in higher plants has demonstrated changes in the over-all enzyme pattern which are characteristic of the micronutrient element in question (112). Although the changes involved increases of certain enzyme activities and decreases of others, only the metallo-enzymes such as polyphenol oxidase, ascorbic acid oxidase, and peroxidase decreased in concentration in plants deficient in the specific metal concerned, i.e., copper and iron, respectively. In the case of all other metal deficiencies, however, polyphenol oxidase and peroxidase were elevated in concentration at least two to six times that of the control. Ascorbic acid oxidase and glycolic acid oxidase doubled in zinc- and manganese-deficient material and DPNH-diaphorase was raised two-fold by copper, manganese, and zinc deficiencies. Manganese deficiency caused an increase in all of the systems measured. While these data corroborate those of other workers with respect to increased activity of polyphenol oxidase with boron deficiency (77, 96, 129) and increases of peroxidase and polyphenol oxidase in zinc-deficient material (13, 121, 128), the results showed that these effects occurred with five different metal deficiencies. In the case of decreases of metallo-enzymes under conditions where the specific metal concerned is deficient in the nutrient medium, the obvious explanation would be failure of incorporation of the necessary element into the enzyme. The possibility is not ruled out, however, of a secondary effect whereby the metal may be indirectly involved in the synthesis or degradation of the protein moiety of the same enzyme. In the case of increased activities of certain enzymes under metal deficiency conditions, the hypothesis already advanced based on alteration of protein metabolism would apply. The increase in enzyme concentration would take place at the expense of other cellular proteins. Direct participation of the metals as inhibitors of those enzymes which increase under deficiency conditions has been experimentally ruled out.

Of the various relationships observed between micronutrient element deficiencies and enzyme content in higher plants, the specific association of a 10- to 30-fold increase in the concentration of isocitric acid dehydrogenase in copper deficient tomato leaves (111) is by far the most outstanding. As already indicated above for other enzymes which increase under various metal deficiency conditions, the effect of copper can probably be ascribed to an alteration of protein metabolism. It is of interest that oxalosuccinate carboxylase activity in extracts of copper-deficient leaves paralleled the observed rise in isocitric dehydrogenase activity, thus providing further support for the possibility that these two activities are catalyzed by the same enzyme (48).

Although it has been shown (112) that such metallo-enzymes as polyphenol oxidase, ascorbic acid oxidase, peroxidase, and carbonic anhydrase (169) are decreased in concentration in plants deficient in the specific metal

concerned, it does not necessarily follow that other nutritive conditions would fail to decrease these enzymes. By the same token it would be fallacious to conclude that a particular enzyme contains a specific metal component simply because a deficiency of the latter results in decreased concentration of the enzyme. At the most such evidence is suggestive. The effect may well be due to an indirect influence on the synthesis of the apoenzyme, as in the case of zinc deficiency on alcohol dehydrogenase and the tryptophan synthesizing enzyme in *Neurospora* (113). Quinlan-Watson (126, 127) has reported a decrease in aldolase of higher plants under conditions of zinc deficiency, whereas a copper deficiency had no effect on the concentration of the enzyme. Far more evidence would be necessary to show this enzyme to be a zinc protein, despite the temptation of analogy with the suggested metallo-nature of aldolase of yeast (162, 163) and *Clostridium perfringens* by Bard & Gunsalus (10) for which iron has been indicated as a component. Earlier studies by Stumpf (152) did not succeed in showing a metal requirement for pea aldolase. This is also true for animals. Although Wood & Sibley (169) obtained a decrease in carbonic anhydrase, a known zinc protein in animals, with a zinc deficiency in oats, they rightfully questioned whether such data were indicative of the zinc nature of the enzyme. They decided that the observed decrease could best be ascribed to an indirect effect on protein formation.

In view of the characteristic enzyme patterns arising specifically from each of the micronutrient element deficiencies (20, 112), it has been suggested (21) that the measurement of some of the metallo-enzymes such as ascorbic acid oxidase, peroxidase, etc., be used as a means of diagnosing deficiency conditions in plants, in some cases prior to the appearance of visual symptoms. However, the important limitation of such a method under field conditions would be the necessity of dealing only with individual metal deficiencies, since different metal deficiencies can affect enzyme concentrations in opposing directions. This type of antagonism could very much complicate enzymatic patterns in the case of multiple metal deficiencies. This is also true in the cases of toxicity of certain metals where very little information is available concerning enzyme patterns.

An interesting effect of a metal deficiency has been reported by Bentley & Phillips (15) who describe a 50 per cent decrease in phosphorylating activity in liver homogenates of manganese-deficient chicks. The addition of Mn^{++}, cytochrome-*c*, and Krebs cycle intermediates *in vitro* had no significant effect on phosphorylation in manganese-deficient homogenates.

Other recent studies of micronutrient element deficiencies on enzyme yield has been the work of Crewther (32) who found that Fe^{++} supplement was necessary to yield maximal activities of hydrogenase and the hydrogenalyses in *Aerobacter polymyxa*. Lulla (93) has reported that iron and manganese were required for maximal production of amylase by *Bacillus subtilis*. The inhibition by aureomycin of the enzymatic reduction of the nitro group of chloroamphenicol and *p*-nitrobenzoic acid to their corresponding amines has been reported by Saz & Shi (138). The reversal of this inhibi-

tion by Mn^{++} further substantiates the metal chelating effect of aureomycin as reported by Albert (3).

It is clear that a great deal of additional work is required on the general metabolic patterns which are evoked under various conditions of trace metal nutrition. Such studies should eventually include not only the deficiency and toxicity effects of essential metals, but the nonessential metals as well.

Concluding Remarks

Several general patterns evolve from the physico-chemical and nutritional studies on the influence and mechanism of action of metals in enzyme systems. Although there are exceptions to these generalizations, it appears that those metals most closely associated with electron transferring systems are copper, iron, and molybdenum. It is clear from nonenzymatic studies that these metals have the inherent capacity to function as electron mediators in oxidation-reduction reactions. The important biochemical questions are: (a) why, in combination with specific proteins, is this capacity for catalyzing oxidation-reduction reactions accentuated, and (b) what is the nature of the linkages which allow specific coupling of these metal systems to others, allowing the transfer of electrons along specific pathways? The pattern which seems to be emerging is that these metals are not required specifically for the combination of substrate to the catalytic protein, but rather function primarily as "electron couplers" from one protein system to another.

It is clear from the tabulated results that magnesium, and to a certain extent manganese, are required primarily for those reactions involving group transfer, in particular those in which phosphate participates. In recent years it has become increasingly clear that enzymes participate intimately in group transfer by serving as the intermediate carrier. Magnesium plays a predominant role in promoting the formation of the enzyme-substrate complex and the resulting intermediate of the reaction. The presence of a pyrophosphate structure in many of the cofactors and substrates involved in group transfer suggests that a chelate structure with magnesium is probable.

The predominant metal involved in general enzymatic decarboxylation and hydrolysis reactions is manganese (and to a certain extent zinc and magnesium). At present there is no general agreement as to the primary mechanism of action of these metals. Certain workers feel that they form an essential structure with the substrate thus acting to bring the substrate into combination with the protein. Other workers feel, however, that the metal combines with the enzyme and functions primarily to accelerate and therefore increase the concentration of an essential intermediate in the reaction. It is evident that manganese does not have strong inherent properties for catalyzing decarboxylation, whereas other metals, such as copper, which do not function as cofactors in enzymatic decarboxylation, are very effective in nonenzymatic reactions. The suggestion, therefore, that manganese functions in enzyme systems by forming chelate structures with the substrate is lacking strong experimental support.

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NUTRITION BY FOLIAR APPLICATION[1]

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Because of the fragmentary nature of the reports bearing on this subject, it will be considered under three main headings: (a) plant responses to sprays of specific nutrients, (b) factors affecting absorption and use of nutrients sprayed on leaves, and (c) conditions determining the feasibility of nutrition by foliar application. Some duplication of reference to particular studies is necessary under these headings. No attempt is made to review all articles pertinent to the subject. Rather, selection of papers for citation has been rigorous in order to permit critical evaluation of the current status of knowledge in the space allotted for this article.

PLANT RESPONSES TO SPRAYS OF SPECIFIC NUTRIENTS
Micronutrient Elements

Iron.—Sprays of iron compounds have been used experimentally over the past three decades on many crop plants in attempts to eliminate iron deficiency chlorosis occurring under alkaline or manganiferous soil conditions.

The earliest successful commercial use of iron sulfate sprays was on chlorotic pineapple fields in the Hawaiian Islands, and resulted from the field experiments of Johnson (1, 2). Under Hawaiian conditions pineapple chlorosis resulted from unavailability of iron due to extremely high manganese in the soil (3), and Johnson recognized the similarity of this symptom to that of lime-induced chlorosis previously reported by Gile in Puerto Rico (4). Johnson found (a) that ferrous sulfate ($FeSO_4 \cdot 7H_2O$) at 2 to 8 per cent in aqueous solution gave temporary recovery from chlorosis; (b) that young plants were more subject to injury from the sprays than older ones, and hence were not safely sprayed at the higher concentration; (c) that the period of effectiveness of a single spray varied with the degree of unavailability of iron in the soil and the vegetativeness of the plant. Thus, with young rapidly growing plants, on soil very deficient in available iron, sprays at low concentration and at intervals of one month might be necessary to hold chlorosis at a low level, whereas with older plants a higher concentration and a longer interval might be quite satisfactory.

The fixation of absorbed iron in the sprayed leaves with little or no translocation to the growing region, and the intolerance of leaves and fruits of most species to high concentrations of iron salts, still remain the greatest stumbling blocks in the way of commercial use of iron sprays on the many crop species which suffer from lime-induced chlorosis and which show temporary responses to such sprays. These problems and the kind of approach

[1] The survey of literature pertaining to this review was completed in October, 1953.

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that has been used by many workers in attempting solutions are illustrated in a study by Guest & Chapman (5). In dipping tests, using orange, grapefruit, and lemon leafy shoots affected by lime-induced chlorosis, more than thirty iron compounds were used at three concentrations, at high and low initial pH, with and without wetting agents; it was found that none of the treatments caused complete recovery and in none of them was there benefit to growth made subsequent to the time of dipping. Some of the treatments caused more complete temporary recovery than others, but none was much more satisfactory than ferrous sulfate at 2 pounds per hundred gallons, in acid solution, with Vatsol OT(dioctyl sodium sulfosuccinate) at 1 pound per hundred gallons. The results of the dipping tests were confirmed in experiments involving entire trees, but it was also found that some necrotic spotting of the rind occurred on young fruits under the treatments most effective in eliminating chlorosis.

This study and the work of others have indicated that although the tolerance of leaves may be somewhat greater to solutions of organic molecules containing iron than to comparable concentrations of such inorganic salts as ferrous sulfate, the recovery from chlorosis may be less. The small quantities of iron in fungicidal applications of such organic materials as ferric dimethyl dithiocarbamate have not caused appreciable recovery from chlorosis. There is now interest in the possibility that sprays of iron chelates may cause improved control of iron deficiency, but no published reports have come to my attention which give promise of this.

Zinc.—The discovery, in 1931, by Chandler (6, 7) that the symptom complex rosette, mottle-leaf, and little-leaf of fruit trees was cured by trunk injections of zinc-containing materials led to widespread experimentation with methods of application of zinc materials to affected trees. As in the case of iron deficiency, soil treatment even with massive doses of zinc salts was frequently found to be ineffective or only partially effective in control of this trouble. There have been variable responses to zinc sprays, but in most species sprays have been found to be more effective and more prolonged in their effects than have iron sprays in the control of iron deficiency. The studies of Parker (8, 9, 10) on mottle-leaf of citrus are examples of investigations with responsive species. The following conclusions drawn from these investigations are of particular interest. (*a*) A single spray of one of several zinc compounds, if applied at a concentration supplying 1.15 pound of metallic zinc or more per 100 gallons of solution, gave complete recovery from mottle-leaf for one to three years. Among the effective materials were zinc sulfate, zinc oxide, zinc carbonate, and zinc sulfide. (*b*) Injury to foliage and fruit resulting fom zinc sprays could be eliminated without loss of beneficial effect by use of a precipitating agent. For this purpose hydrated lime, soda lime, or lime sulfur appeared effective. (*c*) While the use of dusts gave beneficial responses, in general they were not as certain nor as long in duration as sprays which applied equivalent amounts of zinc. (*d*) At low zinc concentrations,

the use of a wetting and adhesive agent sometimes resulted in improved effect of a spray. This was not always true, and at concentrations yielding more than 0.5 pound of zinc per 100 gallons there did not appear to be benefit from such materials. (e) At low concentrations, sprays applied just prior to a growth flush were effective over a longer period than sprays applied in the beginning of a period of vegetative inactivity. This was not true at concentrations yielding 1.15 pounds of zinc or more per 100 gallons.

It should be emphasized that not all species respond so satisfactorily to foliage sprays of zinc. Apple and pear trees are more responsive to concentrated sprays of zinc sulfate (25 pounds per 100 gallons) applied to the dormant branch system just prior to bud opening (11). The use of these dormant sprays also eliminates the hazard of foliage and fruit injuries, which are greater in these species than in citrus. Sweet cherry and walnut usually do not respond satisfactorily to either kind of spray treatment and they are therefore usually treated by injection (7).

Copper.—Copper deficiency of several species of perennial plants has been controlled satisfactorily by foliar sprays. The investigations of Dickey, Drosdoff & Hamilton (12) with young copper-deficient tung trees, and those of Fudge (13, 14, 16) with citrus in Florida, may be used to illustrate such responses. In the studies with tung, it was found that a single spray of copper-lime mixture at 8–8–100 (8 pounds $CuSO_4 \cdot 7H_2O$, 8 pounds $Ca(OH)_2$ and 100 gallons H_2O) in the early growing season prevented development of copper-deficiency symptoms on all vegetative growth produced subsequently in that year. Copper-lime sprays of lower concentration gave temporary control of the symptoms, but it did not last through the season. Sprays of 1 pound of copper sulfate per 100 gallons without equivalent weight of hydrated lime caused foliage injury. In the studies with citrus, it was reported that sprays of copper-lime combinations applied thirty days before bloom prevented development of vegetative symptoms, increased the yield and improved fruit size and quality in the year of application.

From these studies and those with other crops it is evident that copper sulfate is rapidly absorbed by leaves, and that there is translocation of the copper absorbed to growing regions and probably to developing flowers and fruits. The toxicity of copper compounds to leaves when applied even at low concentrations appears to be of a similar order as that of zinc salts, and the prevention of injury by precipitation on the surface of the leaf by reaction with hydrated lime is likewise similar to the situation with respect to zinc.

Manganese.—As in the case of copper deficiency, manganese deficiency of fruit trees is easily controlled by foliar sprays of manganous sulfate ($MnSO_4 \cdot 4H_2O$). Parker & Southwick (15) in California and Camp & Fudge (16) in Florida have found that a single spray of manganese sulfate at 2 to 4 pounds per 100 gallons usually causes complete recovery of chlorotic citrus leaves and prevents development of symptoms on subsequent growth in the year of application. Braucher & Southwick (17) working with manganese-deficient

citrus, and Woodbridge & McLarty (18) working with apple and peach, to mention three other species, have also caused recovery from and control of chlorosis in the year of application, by dilute sprays of manganese sulfate. In general, the tolerance of leaves to manganese sulfate sprays appears to be greater than to zinc or copper sulfate sprays, so that the need for hydrated lime precipitants in solutions containing 2 to 5 pounds of manganese sulfate per 100 gallons of water is questionable. It is of interest that in Florida the use of soil applications of manganese sulfate, zinc sulfate, and copper sulfate is preferred to spray, not because they are as effective or less hazardous than sprays, but because the sprays favor the development of scale insects, which then have to be controlled by insecticides (19). Dormant sprays appear to be less effective in control of manganese deficiency of deciduous trees than in the control of zinc deficiency.

Boron.—Foliar sprays of borax or boric acid have been found satisfactory as a means for controlling boron deficiency of several fruit and vegetable crops. Thus, Askew & Chittenden (20) reported controlling the internal cork symptom of boron deficiency on apple by use of a single spray of hydrated borax at 8 pounds per 100 gallons. This spray increased the boron content of fruit sampled three months later four to five fold. Hill (21) stated that in Canada incipient boron deficiency of celery on acid peat soils may be checked more rapidly by inclusion of borax at 2 to 3 pounds per 100 gallons in fungicidal sprays applied when the crop is from one-third to one-half grown than by soil applications.

Because of the rather narrow range of boron concentration in perennial plants between deficiency and toxicity levels, it has been found desirable, particularly under acid soil conditions, to hold soil applications to rather low rates, and to make them at intervals of several years. In New York, the practice in commercial apple orchards needing boron supplements is to make soil applications of borax at intervals of three years. A comparison by Burrell *et al.* (22) of the residual influence of a moderate soil application of borax with that of two spray applications indicated that the soil treatment had the greater influence on leaf and fruit content of boron in the year after treatment. In the year of treatment, however, the opposite was true. Thus, the absorption of boron by leaves and translocation to fruits may be rapid but there may be little carry-over of effects of a single spray from one year to the next.

Molybdenum.—Of the nutrient elements thus far identified with certainty, it seems probable that molybdenum is required in smaller concentrations than any other. Nevertheless, deficiencies of it have been found in several crop plants, and Stewart & Leonard (23) have found satisfactory response of citrus trees showing deficiency symptoms to a single spray of sodium molybdate at 1 ounce per 100 gallons. The treatment caused regreening of spotted foliage within three to four weeks of application. These workers stated that there appeared to be little translocation of molybdenum from the sprayed lower halves of citrus trees to their unsprayed tops.

MACRONUTRIENT ELEMENTS

While the division between micronutrient and macronutrient elements is an arbitrary one and the terms are subject to criticism, they serve here to stress the fact that the plant requirements for the nutrient elements considered above are smaller than are the requirements for the elements to be considered below.

Nitrogen.—The use of foliar sprays as a means of furnishing a considerable part of the nitrogen needs of several crops has been studied intensively in the past dozen years. Hamilton, Palmiter & Anderson (24), working in three New York apple orchards, sprayed them four times in the early growing season with sodium nitrate, potassium nitrate, ammonium sulfate, and urea respectively added to fungicidal and insecticidal mixtures of sulfur, arsenate of lead and hydrated lime. They reported serious leaf burning from the solutions of nitrate salts at 5 pounds in 100 gallons or higher. Ammonium sulfate at 8 pounds plus 3 of hydrated lime caused leaf injury. At lower concentrations there was no injury when hydrated lime was in the spray solution. The reaction between ammonium sulfate and calcium hydroxide would release the ammonia at the leaf surface so that no nitrogen response could be expected; none was reported. Urea at 5 pounds per 100 gallons plus 1 pound of lime caused no injuries and resulted in increase of leaf chlorophyll and leaf total nitrogen in comparison with leaves from unsprayed trees. Higher concentrations sometimes caused leaf injury.

In subsequent work by Fisher and co-workers (25, 26, 27) it was established that over a period of years application of three urea sprays at a rate of 5 pounds per 100 gallons at weekly intervals in the early post-bloom period gave nitrogen effects sufficient to keep apples moderately vigorous and productive. Comparing the effects of foliar application with those of soil application of urea in the spring, they found that leaf sprays were as effective in promoting tree productivity as, and possibly a little more effective than, soil application of the same amount of nitrogen. At moderate levels of application, leaf sprays of urea made in the period prior to bloom or shortly thereafter frequently inhibited red color development on the fruit a little less than comparable spring soil applications of urea. However, mid-summer urea sprays caused marked reductions in red color development. Fisher (27) concluded that the initial nitrogen effects of urea sprays were more rapid, greater, and shorter in duration than the effects from comparable spring soil treatments. General observations on injuries to apple leaves caused by urea sprays indicated that they were most likely to occur on young developing leaves, and when urea was combined with such water-soluble and caustic fungicides or insecticides as lime-sulfur, dinitro or organic mercury materials.

The commercial use of urea sprays in nitrogen fertilization of the apple has been acompanied by trials with other crops including banana, pineapple, sugar cane, peach, grape, and citrus, as well as some vegetable crops. There is no published information concerning the success with banana and pine-

apple, although the extensive use on those crops in Central America and Hawaii indicates that some promise exists for the practice there. In a progress report concerning foliar fertilization of sugar cane by aircraft, Humbert & Hanson (28) presented evidence that a rapid increase of leaf total nitrogen and leaf chlorophyll followed spraying of sugar cane with concentrated urea solutions after the fields had "closed in". This increase was much more rapid than that caused by comparable soil treatments. The early difference between airplane spray and soil treatments lasted for only four weeks or less, the period being inversely related to the rapidity of growth by the cane plants. As much as 67 pounds of nitrogen per acre was applied in a single spray without injury, provided the plants were high in moisture at the time of application. This contrasts with a maximum safe rate of application of about 15 pounds per acre for apple trees in New York. Whether or not a urea spray caused increase of yield appeared to depend on the initial nitrogen status of the plants and the residual available nitrogen in the soil at the time of spraying.

In general, the experiences of investigators with urea spraying of the peach and grape have been disappointing or inconclusive. Weinberger, Prince & Havis (29) reported that two varieties of peach at Fort Valley, Georgia, sprayed three times with urea at 5 pounds per 100 gallons in the early postbloom period showed no significant response to the treatments, either in terms of tree behavior or leaf total nitrogen. Proebsting (30) in California stated that peach trees which were very deficient in nitrogen showed no response to as many as six applications of urea solution at 5 pounds per 100 gallons. Although Bullock & Benson (31) obtained direct evidence of nitrogen absorption by urea-sprayed peach leaves under greenhouse conditions, they failed to find significant effects of three sprays at 5 pounds per 100 gallons on yield or fruit maturation dates under orchard conditions. These results are similar to those reported for Labrusca grapes. Working with Concord grapes, Mack & Shaulis (32) noted an increase in leaf chlorophyll due to leaf sprays of urea, but Fleming & Alderfer (33) found that one or two mid-summer sprays of urea at 5 pounds per 100 gallons failed to improve vigor or yield of a vineyard receiving good care and adequate soil applications of commercial fertilizers containing nitrogen.

Judging from the preliminary reports of three workers in southern California (34, 35, 36), the leaves of lemon and orange trees are efficient in absorption of urea sprays, and there is a more rapid increase of leaf nitrogen as a result of such sprays than as a result of comparable applications of nitrogen to the root medium. Growth of young trees under greenhouse conditions was stimulated by urea sprays. The quality of fruit on bearing grapefruit and orange trees was not measurably affected by six urea sprays which caused a 25 per cent increase in leaf nitrogen. Leaf injuries sometimes resulted from such sprays; the higher the concentration above 1 per cent by weight, the greater the tip and marginal yellowing and necrosis. The injuries were reduced or eliminated by additions of sucrose to the spray mixture, as first sug-

gested by Emmert & Klinker (37), but absorption of urea was reduced also.

Working with tomatoes under field conditions Mayberry & Wittwer (38) found that there was a significant hastening of the time of ripening and a possible slight increase in total yield due to four urea sprays at 0.75 per cent; greenhouse tomatoes sprayed four times with 0.50 per cent urea also yielded significantly more than unsprayed plants. Under field condititions these authors found a significant increase in the yield of celery sprayed five times with 1 per cent urea solutions. On the other hand, Brasher, Wheatley & Ogle (39) did not find significant increase of yield due to urea spraying in field plots of tomato, lima beans, potato, cantaloupe, or cucumber to which standard soil applications of fertilizer containing nitrogen had previously been made. These authors did find significant increases in tomato yields to result from 11 sprays of urea in an experiment in which no fertilizer was applied to the soil of the unsprayed check plots, but they obtained greater yield increases at less cost from plots in which nitrogenous fertilizer was applied to the soil.

From these and other studies it may be safely concluded that nitrogen from urea is readily absorbed by the leaves of many diverse species, and under some conditions sprays of this material may be relied on as an important source of nitrogen.

Magnesium.—Experiments in New York (40), Massachusetts (41) and Maine (42) have indicated that three to four sprays of 2 per cent Epsom salts $(MgSO_4 \cdot 7H_2O)$ during the early post-bloom period are effective measures for preventing the development of magnesium deficiency symptoms of apple, in the year of application. There appeared to be little or no carry-over of the effect of the sprays from one season to the next. The Maine workers (42) and subsequently those in New York have found that at least three sprays are necessary for commercial control. Commercial responses by apple trees to soil applications of Epsom salts, dolomitic limestone or kieserite are usually delayed by one to three years. Scott & Scott (43, 44) found that magnesium deficiency of several eastern bunch grape varieties could be prevented by two drenching sprays of 0.4 per cent Epsom salts or 2 pounds of the same material applied to the soil. There was visible response to the spray applications by some of the vines about six weeks after application, whereas there was a one year lag in response to the soil treatment. Working with greenhouse tomatoes, Nicholas (45) found five foliage sprays of 2 per cent Epsom salts gave control of magnesium deficiency. This treatment was more rapid and more efficient in its effect than calcined kieserite applied to the soil. Heymann-Herschberg (46) was able to cause satisfactory recovery from magnesium deficiency of Shamouti orange in Palestine by application of two to four sprays of Epsom salts at 4 per cent. Soil applications of magnesium salts gave no benefit.

From these and other studies, it is safe to conclude that magnesium in Epsom salts may be absorbed by the foliage of a wide range of species in quantities sufficient to prevent development of deficiency symptoms on plants that would otherwise have shown them. The fact that field responses are

somewhat slower than those to urea sprays leads to speculation as to whether leaf absorption of magnesium from Epsom salts is as rapid as absorption of nitrogen from urea. There have been no reports of studies comparing the relative rates of absorption of these two elements, but the study of Montelaro, Hall & Jamison (47), indicating that leaf injury to tomato plants from urea sprays was reduced by addition of Epsom salts to the spray solution, suggested that there may have been an effect of the divalent ion on the mechanism of absorption which resulted in a net reduction in intake of both nitrogen and magnesium.

Phosphorus and potassium.—In spite of the fact that phosphorus and potassium together with nitrogen are the nutrient elements most commonly supplied in fertilizer supplements, there have been relatively few reports of responses to foliage applications of phosphorus and potassium in separate sprays. From those that are published, it appears that both nutrients may be absorbed by leaves to which they are applied, and that responses may be expected in proportion to the deficiency below the plant requirements at the time of spraying on the one hand, and the amount absorbed on the other.

Thus, Silberstein & Wittwer (48) compared the effects of foliar application of a number of solutions of inorganic and organic phosphorus compounds on growth of corn and tomato plants under greenhouse conditions. They found that several of the treatments caused significant increases in growth over that produced by plants not supplied with phosphorus. Ortho-phosphoric acid at 25 mm./l. was among the best treatments. However, phosphorus added to the nutrient medium around the roots gave greater growth responses than any of these treatments. Under field conditions, a total of 2.73 pounds of P_2O_5 as orthophosphoric acid sprayed on tomato plants gave a greater early yield response than did 135 pounds of P_2O_5 as superphosphate applied to the soil. The total yield of tomatoes at the end of the experiment was increased 12 per cent over untreated plants by the spray application and 24 per cent by the soil application. Thus, it seems likely that the early advantage of the spray treatment was due to more rapid absorption and metabolism of the phosphorus applied, but that the supply over the total period was not great enough to maintain the lead over plants to which superphosphate was supplied. Auto-radiograms of corn and bean plants which had been treated with foliar applications of ortho-phosphoric acid containing radioactive P confirmed the conclusion that absorption of P applied to leaves is very rapid, and that the absorbed phosphorus moves promptly to the growing regions.

Although the studies of Eggert, Kardos & Smith (49) with apple trees did not permit evaluation of growth responses, they demonstrated clearly that several water-soluble phosphorus salts containing P^{32} were promptly absorbed by apple foliage and translocated to other parts of the tree. Di-ammonium phosphate appeared to be absorbed in greater amount than four other inorganic phosphorus salts under the conditions of their experiments.

The studies of Burrell and co-workers (50, 51) with apple trees showing

potassium-deficiency leaf scorch in the Champlain Valley of New York have given evidence that this trouble may be prevented by the application of five to six sprays of potassium sulfate at 1 per cent, as well as by soil applications of the same salt or potassium chloride. The spray treatments did not raise the potassium levels of the leaves as much as did rather heavy soil treatments with soluble potassium salts. Some leaf injury occurred as a result of the sprays.

Sulfur.—I have failed to find reference to positive nutritional responses of plants to the spraying of sulfur. Yet it is well established by the studies of Thomas and his co-workers (52) that the sulfur from SO_2 fumes is readily absorbed by the leaves of many plants, and that metabolism and translocation of the absorbed sulfur takes place with considerable efficiency. Turrell (53, 54) likewise, in his studies of sulfur toxicity to citrus, has demonstrated that elemental sulfur dusts and spray deposits applied as pesticides are to some extent absorbed by leaves and fruits and metabolized by them. The injuries to leaves caused by several sulfur fungicides are evidence of absorption of them by the leaves. It should be safe to expect, therefore, that, under conditions of sulfur deficiency, sprays of soluble sulfates would have essential nutritional value and cause growth responses.

FACTORS AFFECTING ABSORPTION AND USE OF NUTRIENTS SPRAYED ON LEAVES

ABSORPTION[2]

Contact angle and surface wetting.—While ultimately aqueous solutions of nutrients sprayed on leaves must penetrate living cells in order to be metabolized by them or translocated within the plant, the initial entry may be via the stomatal apertures to the cells of the mesophyll as well as directly through the cuticle and into the cells beneath it. In order for penetration of cuticle or stomates by a liquid to occur, the leaf surfaces must first be wetted. The ability of a liquid to wet a solid surface is a function of its contact angle on the surface; this depends on the surface tension of the liquid and on the nature of the solid surface.

Several workers have reported measurements of the contact angles of liquids on leaf surfaces. Fogg (55) found great differences in the contact angles of water on leaves of different species, differences due to age of leaf and diferences due to leaf water content. These differences seemed to be due to two major variables: (*a*) variations in surface conformations and pubescence, and (*b*) variations in the composition of the cuticle. Ebeling (56) determined experimentally that the addition of wetting agents reduced the contact angle of water on leaf surfaces to that of the heavier insecticidal oils. He found also

[2] In preparing this section I have been assisted by the Doctoral Thesis presented to the Faculty of the Graduate School of Cornell University in 1951 by Dr. James A. Cook, Division of Viticulture, University of California, Davis, California.

that the rise of water in small waxed glass capillaries was increased by addition of wetting agents; this resulted from reduction in surface tension and contact angle.

Ursprung (57) tested the wettability of the exposed parenchyma of leaves from a number of species and found that there was little or no wetting by water. Lewis (58) found that when leaf strips were suspended with the lower cut edge in various liquids, benzene, chloroform and olive oil rose 2 centimeters in the intercellular spaces within sixty seconds, and paraffin oil rose 0.5 centimeter in five minutes. Water did not rise at all until a little sodium taurocholate was added; then it rose the same distance and rate as paraffin oil.

These and similar studies suggest that some of the reported variations in absorption of aqueous solutions by leaves may have been the result of differences in wetting of the external surface due to the characteristics of the cuticle, and some may have been due to differences in surface tension of the solution. In view of the disappointing results from urea sprays on peach trees, the studies of Hesse & Griggs (59) are interesting. These authors found considerable differences in the degree of surface wetting of peach leaves of different varieties, which appeared to be due to the composition of the cuticle. The variety Elberta was classified in the group of varieties with leaves having least wettability.

The contribution of a wetting agent in increasing the efficiency of absorption by leaves is indicated in the studies of Cook & Boynton (60) and Guest & Chapman (5). The former, working with McIntosh apple leaves, found that absorption of urea solutions by lower leaf surfaces in four hour periods was increased, on the average, more than 100 per cent by the addition of Tween 80 (a sorbitan mono-oleate polyoxyalkylene derivative) at 0.1 per cent or Tween 20 (a sorbitan monolaurate polyoxyalkylene derivative) at 0.01 per cent. The effect of the detergent in increasing absorption was apparent only when the leaves had not been sprayed previously with solutions containing wetting agents or oils. The latter investigators, working with citrus, found that the recovery of leaves from lime-induced chlorosis was more complete when Vatsol OT or another wetting agent was added to the iron sulfate spray solution. Lacking the detergent, the leaves showed only stipled recovery, indicating incomplete absorption of the nutrient solution.

Paths of entry.—In a study designed to determine the paths of entry of spray materials into McIntosh apple leaves Roberts, Southwick & Palmiter (61) seemed to find that the cutin of the external leaf surface was in discontinuous lamellae parallel to the outer epidermal wall. Pectinaceous substances, staining a reddish-pink with Ruthenium Red, were found in intermittent layers in the outer epidermal walls and interspersed with the cutin lamellae. The pectinaceous materials, which should have great water absorbing power, formed a continuous path from the layers in the cuticle through the anticlinal walls of the epidermal cells to the cell walls of the vein extensions and bundle sheaths surrounding the larger veins of the leaves.

Thus, there appeared to be paths by which aqueous solutions could enter through the cuticle to the living cells surrounding the vascular tissues. A study by Palmiter *et al.* (62) using microchemical techniques demonstrated that indeed these paths do carry solutes into the leaves.

The degree to which and conditions under which aqueous solutions may enter the stomates of leaves and diffuse throughout the intercellular spaces of the mesophyll are not yet adequately understood. But despite the contention of Crafts (63) that such absorption through stomates is negligible due to physical considerations, there seems to be an increasing body of circumstantial evidence to indicate that the stomates are frequently the most important initial paths of entry of nutrients in solution, even though entrance through the cuticle or uncutinized guard cells may also be important over a longer period of time. In considering the relative significance of these two paths of entry, the work with species which have stomates only on the lower leaf surfaces is of particular interest. Apple (64) and citrus (65) are two such species.

Ginsberg (66) observed that a number of insecticidal oils penetrated the lower surfaces of apple leaves rapidly, the rate of penetration varying inversely with the viscosity of the oil. On the upper surfaces only the lighter oils reached the leaf tissue. Kelley (67) applied oil emulsions containing as little as 1.5 per cent oil to the upper and lower surfaces of Grimes Golden apple leaves. He found, by free-hand sectioning, an abundance of oil emulsion in the intercellular spaces of leaves whose lower surfaces were sprayed but none in the intercellular spaces of leaves whose upper surfaces were sprayed.

Cook & Boynton (60) compared the rates of absorption of urea solutions sprayed on the upper and on the lower surfaces of McIntosh apple leaves over various time intervals from 2 to 72 hr. They found that although efficiency of absorption varied greatly from run to run, the lower surfaces always absorbed a larger proportion of the urea applied than did the paired upper surfaces. A representative experiment in which they determined the percentage of applied urea nitrogen that was absorbed after 2, 8, 24, and 72 hr. demonstrated that whereas 42 per cent of the nitrogen applied to lower leaf surfaces was absorbed within 2 hr., it took more than two days for the upper surfaces to absorb that proportion of the nitrogen applied. At the end of three days in this experiment the upper surfaces had absorbed 49 per cent of the urea nitrogen sprayed on them and the lower surfaces had absorbed 85 per cent of that applied to them. Thus, the ratio of percentage absorption by lower surfaces to percentage absorption by upper surfaces narrowed with time, and extrapolation of the time curves for the percentage absorption by the two surfaces indicated that they both would approach 100 per cent absorption after six or seven days had elapsed. In other words, the shorter the time interval, the greater the relative efficiency in absorption by lower leaf surfaces; the larger the time interval, the smaller the advantage of the lower surface application, until ultimately none existed. This probably explains the results obtained by Rodney (68) who found that applications of urea sprays to either upper or lower leaf surfaces of young apple trees caused sim-

ilar increases of total nitrogen in leaves sampled 28 days after the last spray application. He concluded that in both cases the urea entered directly through the leaf cuticle since there are no stomates on the upper leaf surface of apple. While his studies indicate that indeed there must be important absorption directly through the cuticle, they give no information on the initial absorption via the stomates by the leaves sprayed on the lower surfaces. However, it may be fairly argued that the work of Cook & Boynton (60) and other similar studies throw no direct light on the degree to which stomates are involved in initial absorption of aqueous solutions by leaves, and the conclusion that the rapid absorption that occurs could only take place through a pore system is at best based on circumstantial evidence. It is also a fact that the lower surfaces of apple leaves are very different from the upper surfaces in many respects beside the presence of stomates: they are reticulate with small veins which protrude slightly, they are pubescent in varying degree, and the cuticle is relatively thin. While it is more difficult to wet lower surfaces of apple leaves than upper ones, they may retain almost twice as much water per unit surface, due to the presence of the reticulations and pubescence.

Working with citrus leaves, Knight, Chamberlain & Samuels (69) found that insecticidal oil applied to the lower surfaces "pours through the stomates" within an hour, and that oil applied to the upper surfaces penetrated the cuticle but did not pass beyond the epidermal cells within the first hour after application. Rohrbaugh (70) found that oil and also a 2 per cent oil emulsion penetrated the lower leaf surfaces of citrus leaves largely through the stomates, spreading in fan-like areas from them. Turrell (54) stated that from consideration of contact angles and capillarity little or no penetration of rain water should occur through stomates of citrus leaves but that sprays with water containing appropriate wetting agents could be expected to penetrate by way of the stomates. Guest & Chapman (5) reported better recovery by citrus leaves showing lime-induced chlorosis when they were sprayed on their lower surfaces with solutions of iron compounds containing detergents than when the upper surfaces were sprayed with these solutions.

If these citations do not give a clear picture of the paths of entry of nutrient sprays into leaves, they at least indicate the present state of knowledge. It may be tentatively concluded that aqueous solutions both penetrate the leaf through the cuticle and make initial entry into the leaf by way of the stomates. While the latter may be of greatest significance in short periods of time, the former may be as effective a means of entrance over longer times.

Temperature and humidity.—Variations in temperature and vapor pressure deficit should influence absorption of nutrients by leaves in so far as they affect the rate of drying and the opportunities for establishment of a liquid film at the leaf surface. Cook & Boynton (60) found that there were significant linear correlations both between air temperature and absorption and between relative humidity and absorption in 42 experimental determinations during

which they were studying the effects of various factors on absorption of urea solutions by apple leaves. Both of these correlations were negative, of course, and indicate that when relative humidity and temperature combine to decrease the vapor pressure gradient at the leaf surface greater absorption may be expected. It should be emphasized that undoubtedly these relationships involve more than the initial effects of vapor pressure gradient on visible drying. Absorption of nutrient sprays takes place over considerable periods of time, and occurs when the leaf surface appears to be dry. It may well be that thin aqueous films resulting from transpiration are frequently more important in promoting absorption of nutrient sprays than is the water of the solution originally sprayed on the leaves.

Age and nitrogen status of absorbing leaves.—The studies of Cook & Boynton (60) indicated that the lower surfaces of McIntosh apple leaves which were grown under high nitrogen conditions were more efficient in absorption of urea nitrogen in a two-hour period than were low nitrogen leaves. Similarly they found that lower surfaces of apple leaves basal on shoots, and therefore older, were less efficient in short-period absorption of urea nitrogen than lower surfaces of leaves close to the terminals of the same shoots, and therefore younger. In the latter study it is worth noting that there was no significant difference in absorption by upper surfaces of the leaves of different ages, although in six out of ten cases the absorption by the upper surfaces of the older leaves was greater than that by the younger leaves.

It seems clear that correlations of this sort may not be dealing with causal relationships, but may in fact be the result of physical or morphological differences in the leaves of the different age or N-status categories that are not necessarily determined by age or nitrogen status alone. Thus, the leaves from the trees of higher nitrogen status were somewhat greater in cross-sectional area, among other things, than the leaves of the lower nitrogen trees. The older basal leaves on the shoots used for the leaf age study were thinner in cross section than the younger terminal leaves. The cross-sectional area of leaves must determine their internal exposed surface to some degree. Pickett & Birkeland (71) have found that the internal exposed surface of apple leaves is also determined by the development of the palisade layer which may be affected by the fungicidal spray materials used during the time that the leaves were developing. It does not seem unlikely that the internal surface of the leaf may sometimes limit absorption of nutrient sprays through the stomates, and the correlations discussed above may well have been partly the result of variations in internal exposed surface. The external surfaces of leaves also vary with many conditions in addition to age and nitrogen level. Since the cuticle is the first barrier to absorption through epidermal cells or their walls, the degree to which it is continuous must affect the rapidity with which solutions penetrate the leaf surface directly. Thus, discontinuities caused by weathering, insects, diseases, or pesticidal sprays may often be dominant factors affecting absorption of nutrient sprays under field conditions.

On the other hand, there are some obvious morphological features of young expanding leaves which may make them somewhat more efficient in absorption of solutions than are older leaves, provided they are completely wet by the solutions. In the case of apple leaves, for instance, MacDaniels & Cowart (64) found that the stomates are developed very early, and that the guard cells reach their maximum size while the surrounding epidermal cells are no more than a fifth of their final diameter. Thus the proportion of the lower surface area occupied by stomates is larger in the immature than in the fully expanded leaf. Unicellular hairs initially are present on both surfaces of the developing leaves and occupy a larger proportion of the lower surface on partly expanded leaves than on mature ones. There are also glandular structures on the upper sides of the main veins and at the tips of the serrations; these disappear from mature leaves, as do the hairs from the upper surfaces.

Chemical composition of the nutrient spray.—Although the studies concerned with this subject are fragmentary and do not permit quantitative comparisons, it seems obvious from fundamental considerations that there must be differences in the rate of absorption of nutrient elements sprayed on leaves—differences that are related to the solubility of the compounds containing them, the ionization and activity of the molecules in relation to the absorbing cells, and the mixtures of chemical compounds applied in the spray. We have already seen in the studies of Parker (8, 9, 10) with zinc deficiency of citrus, and those of Dickey, Drosdoff & Hamilton (12) with copper deficiency of tung, that addition of lime to zinc sulfate and to copper sulfate sprays was made in order to cause precipitation on the surfaces of the leaves. On the one hand, with such nutrients the quantity needed to prevent deficiencies is very small; on the other hand, injury to the leaves may result from absorption of large quantities. Hence the addition of the lime as a "safener" is a means of insuring slow absorption. The relative rate of absorption of sprays of iron compounds is difficult to evaluate since there are problems of intercellular and intracellular immobilization (5).

A comparison of field responses to urea sprays and to Epsom salts sprays leads to the conclusion that the former are more rapidly and efficiently absorbed than the latter. There are several kinds of observation that lead to this conclusion. Magnesium is present in apple leaf tissues in less than half the concentration in which nitrogen is found there (72, p. 19). Yet it is necessary to supply more magnesium, in terms of pounds per tree, in the four Epsom salts sprays to insure against development of deficiency symptoms than nitrogen in the three urea sprays which are usually sufficient to keep the tree in an adequate state of vigor (26, 40). The visible responses of apple trees to urea sprays, in terms of increased chlorophyll content of leaves, are often somewhat more rapid than are the visible responses to Epsom salts sprays in terms of prevention of the development of interveinal chlorosis (24, 40). Apple leaves appear to be less subject to injuries from Epsom salts

sprays of relatively high concentration than from sprays of urea at comparable molar concentrations. This is also true for tomato leaves (47).

Beside these observations, it may be significant that Montelaro, Hall & Jamison (47) found that injury by urea sprays at 0.3 M to 0.5 M concentration to leaves of tomato could be greatly reduced by the addition of $MgSO_4$ (as Epsom salts) at 0.15 M to 0.90 M concentration. Conversely, an unpublished study by David Walker at Cornell University has indicated that the addition of urea to Epsom salts sprays in a McIntosh apple orchard showing magnesium deficiency resulted in improvement of control of the difficulty over that obtained with Epsom salts sprays containing no urea. Thus, it seems possible that not only are urea sprays absorbed more rapidly than sprays of Epsom salts, but also that each of these compounds influences the absorption of the other when the two are mixed in the spray solution.

The facts that Haas (34) was able to eliminate injury to lemon foliage caused by high concentrations of urea when he added lime to the solution and that Mack & Shaulis (32) reduced urea injury to grape leaves by combining the urea with bordeaux mixture (copper sulfate plus hydrated lime), suggest that the calcium and magnesium salts may have reduced absorption of urea as a result of divalent ion effects on permeability.

Emmert & Klinker (37) working with tomato, Kuykendall & Wallace (36) with citrus, and Cook & Boynton (60) with apple have found that the addition of sucrose to a urea spray of injurious concentration delayed or eliminated the leaf injuries that occurred in the absence of the sucrose; the two latter studies gave evidence that the reduction in urea injury caused by the presence of sucrose in the solution was associated with reduction in absorption of urea nitrogen. While this may well have been a causal relationship, it should be borne in mind that direct leaf absorption of sucrose sprays and vegetative responses to them by tomato have been demonstrated recently by Went & Carter (73) and Smith & Zink (74). It is possible that part or all of the sucrose effect in reducing urea injury may have been the result of changes in metabolism of the leaf cells that resulted from sucrose absorption and made them less subject to such injury.

The citations above must suffice as illustrations of some of the more obvious effects of the chemical composition of the nutrient spray on absorption. There is great need for careful quantitative studies in this general area.

Losses of nutrient sprays to the atmosphere and soil.—Thus far, no consideration has been given to the proportion of loss "twixt cup and lip" that may be expected to occur when nutrient sprays are applied under field conditions. Such losses may be of three kinds: (*a*) those due to failure of the spray to reach the leaf surface, (*b*) those due to drip from the leaves, and (*c*) those due to volatilization.

Losses due to failure of the spray to reach the leaf surface or due to drip vary greatly in relation to several circumstances: (*a*) the degree to which the spray equipment used limits its application to the leaf surface, (*b*) the excess

of solution applied over that necessary to wet the leaves completely, (c) the period of time that elapses between spraying and rains or dews which wash the leaves, (d) the degree to which rainfall washes the leaves when it occurs, (e) the adherence and solubility of the nutrient compound on the outer surface of the leaf. The losses of completely soluble compounds like urea and magnesium sulfate from failure to reach the tree and from drip, when they are applied as dilute sprays by modern fixed outlet spray equipment (75) to apple trees, may be as high as 50 per cent. At the other extreme, in the case of the concentrated solutions of urea applied by aircraft to closed-in sugar cane fields, this kind of loss to the soil reported by Humbert & Hanson (28) was no more than 25 per cent and was usually less than 10 per cent. Judging by the work of Cook & Boynton (60), a heavy rain falling within 8 hr. of a urea spray could wash off 80 to 90 per cent of the urea that had remained on the upper surfaces of leaves immediately following spraying, and 40 to 60 per cent of that remaining on their lower surfaces immediately after spraying. Some unpublished studies of Epsom salts absorption by the author indicate that at 8 hr. following spraying the loss due to a heavy rain would be greater than that of urea. On the other hand, if no rain fell for a week, the loss of soluble nutrient compounds due to washing would be small. In the case of species like banana, pineapple, and sugar cane, which have stomates on both upper and lower leaf surfaces, it is possible that the loss of soluble materials due to washing after 8 hr. would be smaller than for species like apple and citrus. Nutrients lost to soil, of course, are subject to recovery by root absorption if there is no fixation there, but the advantage of leaf absorption is gone, if there was any.

There has been some anxiety about possible losses of ammonia to the atmosphere from urea sprays as a result of urease activity at the leaf surface, but thus far no experimental results indicate that these are serious. Harley, Moon & Regeimbal (76) found urease in the leaves of the apple and peach, but under the conditions of their tests the maximum losses of nitrogen due to ammonia volatilization was 4 per cent of that applied. Cook & Boynton (60) were not able to detect any ammonia release to the atmosphere from the surfaces of apple leaves sprayed with urea.

Use

The many positive responses of plants to sprays of nutrients, some of which have been discussed above, are in themselves evidence that following leaf absorption nutrient elements may be effectively metabolized and translocated within the plant. As yet, however, there are rather few studies of the course of metabolism and the conditions affecting translocation of nutrients applied as sprays to leaves.

Boynton, Margolis & Gross (77) studied the metabolism of urea nitrogen sprayed on the lower surfaces of McIntosh apple leaves. In one experiment they investigated the trends of nitrogenous constituents within the leaves

over a period of four days following urea spraying. They found, with leaves which absorbed half the applied urea in 8 hr. and 88 per cent of it in the four day period, that the total nitrogen content increased with time, accounting for 47 per cent of the absorbed nitrogen at the end of 96 hours. The concentrations of the soluble nitrogen fractions and of protein nitrogen varied in different ways over the four day period. Urea N, ammonia N, amide N (exclusive of urea), and alpha amino N reached maximum levels within 8 hr. or shortly after then, and subsequently decreased in concentration with time. Protein nitrogen did not increase until after more than 8 hr. had elapsed, but by the end of four days accounted for 35 per cent of the absorbed N. Thus, about half of the absorbed nitrogen was not present in the leaves at the end of the experiment and was assumed to have been translocated elsewhere. In a subsequent experiment in which the direction of nitrogen translocation from median shoot leaves sprayed with urea was studied, an increase of soluble nitrogen was found in the younger leaves close to the apical growing point, but not in the older basal leaves of the shoot after 24 hrs. In another experiment, the effects of urea solutions of 1 per cent and 3 per cent concentrations applied to lower surfaces of mature McIntosh leaves were studied. The dilute spray caused no visible injury, but at 16 hr. after application some of the leaves treated with the more concentrated spray showed a pinpoint necrosis. Analyses of leaves sampled at 24 hr. after treatment showed the following points of interest. 88 per cent of the applied urea was absorbed by the leaves of both treatments. Thus, three times as much urea was absorbed by the leaves sprayed with the higher concentration than by those sprayed with the lower concentration. There was considerable total nitrogen increase in the leaves of both treatments and this was due to increases in urea, ammonia, amide (less urea) and alpha amino N. For all these fractions the level was higher in the leaves sprayed with the more concentrated urea solution. But the two most striking differences were (a) a level of urea more than ten times as great in the leaves sprayed with 3 per cent solution than in those sprayed with 1 per cent solution, and (b) a sharp drop in protein nitrogen of leaves receiving the higher concentration.

Hinsvark, Wittwer & Tukey (78) tested the degree of injury by solutions of different urea concentration to six species of crop plants grown under greenhouse conditions. They found that cucumber leaves were subject to injury from lower concentrations of urea than bean, tomato and corn, which in turn were subject to injury at lower concentrations than celery and potato. These workers sprayed urea containing C^{14} on leaves of these species and periodically determined the radioactivity of carbon dioxide absorbed from air circulating in a closed system in which the sprayed plants were growing. They found that the radioactivity was greatest immediately after treatment and decreased with time following treatment. Carbon dioxide from the cucumber chamber had the highest initial radioactivity and the shortest period of activity; carbon dioxide from the celery and potato chambers had the low-

est initial activity and the longest period of activity. The addition of sucrose
to urea solution applied to tomato leaves resulted in lower initial activity and
a longer period of activity. From these data the authors have concluded that
plants most easily injured are those which have the highest urease activity.
In drawing this conclusion, the authors assumed that the release of C^{14} to the
atmosphere was initially the result of urease activity which hydrolyzed the
urea, the injury associated with high C^{14} activity in the air being due to ac-
cumulation of ammonia in toxic concentration within the absorbing cells
prior to its conversion to less toxic protein precursors. The effect of sucrose in
decreasing leaf injury was thought also to be due to inhibition to urease
activity. This is a plausible conjecture but direct evidence on urease activity
and ammonia concentration is needed before it can be taken seriously.

Eaton & Ergle (79), in the course of a critical study of the nutritional
interpretation of boll shedding in cotton, sprayed cotton plants at different
times during the growing season with 20 per cent sucrose solution, 1 per cent
urea solution and a mixture of the two. The urea solution caused leaf injury
when applied alone, but in combination with sucrose there was no injury.
The urea application alone, however, caused markedly greater increase in
total nitrogen of the plants than did the combined spray. The total nitrogen
gains due to the urea spray were both in the soluble and insoluble (protein)
fractions. The urea spray alone also decreased the organic acids, particularly
the malic acid fraction, of the leaves. In these experiments there was no
benefit in terms of increased boll retention from the urea treatment; in fact,
the opposite sometimes occurred.

Kuykendall & Wallace (36) found that the root weight of rough lemon
trees in low nitrogen nutrient culture was greatly increased as a result of
urea sprays on the foliage. This was not true in the case of trees whose roots
were in a high nitrogen nutrient medium.

The studies of Silberstein & Wittwer (48) with corn and peas and Eggert,
Kardos & Smith (49) with apple trees have already been discussed in connec-
tion with sprays of phosphorus compounds, and it need only be said that by
use of radiophosphorus in these studies it was demonstrated that phosphorus
absorption by leaves and translocation from them to the growing points of
top and root system may be rapid; the rate appears to depend on the phos-
phorus compound to some extent.

The field studies with iron sprays on various crops give clear evidence of
immobilization within the leaf. Not only is there practically no translocation
from the sprayed leaves, but more often than not the leaf itself does not re-
cover from chlorosis uniformly, indicating a localized immobilization within it.
The studies of Biddulph (80) using Fe^{55} with bean plants illustrate this prob-
lem. He found precipitation of iron as phosphate in the veins of the leaves
and interveinal chlorosis when the pH of the nutrient medium was 7 and
phosphorus was high. When the pH of the nutrient medium was 4 and phos-
phorus was low, radioiron added to the external nutrient solution was rather
uniformly distributed throughout the plant and there was no chlorosis.

Although the above investigations have yielded useful information concerning the metabolism and translocation of nutrients absorbed by leaves, they are not comprehensive enough to give more than general reassurance that nutrients absorbed by leaves may be metabolized by them and translocated throughout the plant. In the case of urea sprays, much more work is needed before there will be an adequate understanding of the course of metabolism in leaves and the causes for leaf injury when it occurs. As for the other nutrients, there has been even less serious work in this area to date.

CONDITIONS DETERMINING THE FEASIBILITY OF NUTRITION BY FOLIAR APPLICATION

A recapitulation of the foregoing discussion in terms of the practical problems of crop nutrition must give due weight both to the promise of this method of application and to its inherent limitations. The usefulness of foliar application of nutrients depends on the following circumstances: (*a*) The existence of special problems that may not be coped with as well by application of the fertilizer to the soil or by soil management. (*b*) Satisfactory plant responses to the nutrient spray. These are mostly determined by the amount of the nutrient required by the plant, the efficiency of foliar absorption and use, and the tolerances of the leaves to the nutrient compounds available for use. (*c*) Economic materials and methods of application.

Special problems.—The special problems of nutrition that create an interest in foliar application are of several kinds. Rapid fixation of nutrients on the soil in forms unavailable to crop plants is one kind of special problem. This is the basis for the use of foliar sprays of iron, zinc, manganese and copper compounds in those situations when sprays are used in preference to soil applications. Another similar special problem is slow response to soil applications and need for a temporary method of control in the period before the soil treatments take effect. This is the main reason for use of Epsom salts sprays on apple trees (81). In that case, the basic problem is a reestablishment of satisfactory levels of calcium and magnesium in the soil, but since soil applications of lime and magnesium salts usually do not cause commercial control of magnesium deficiency in less than three years, spray treatments of Epsom salts are used as a temporary control measure in the interim in those years when a heavy crop is developing on the trees.

The use of a foliar spray of a nutrient as a means of checking development of a deficiency symptom very soon before the trouble is expected or immediately after it has appeared is sometimes a practical means of preventing serious losses. This sort of emergency use has been made of boron sprays on apples. Since the development of boron-deficiency symptoms in marginal situations is usually a dry weather trouble, growers whose orchards have a boron-deficiency history are on the lookout for it in such years and may supplement their triennial soil treatments with an early summer foliage application.

Urea spraying has been of particular interest to apple growers as a means

of controlling the nitrogen effects on tree productivity and fruit quality (82). In so far as it furnishes a means of adjusting the nitrogen level of the tree in accordance with the seasonal conditions that affect these things, it has been useful for this purpose. With sugar cane, on the other hand, the main interest in urea sprays appears to result from the fact that it is impractical to make soil applications of nitrogenous fertilizers during the final period of growth of the crop when nitrogen supplements are sometimes needed (28).

Satisfactory plant responses.—Nutrient elements needed in smallest quantity should lend themselves best to this method of application if they are not immobilized in or near the absorbing leaves. This appears to be the case. Sprays of manganese, copper and boron compounds seem to be readily absorbed and transported in all the crop plants investigated thus far, and one or two dilute sprays give satisfactory responses in a single year. Sprays of zinc compounds likewise appear to be readily absorbed and transported in many species, and a single dilute spray usually gives satisfactory response in the year of application in those species. But there are some in which the zinc appears to move from the point of absorption with difficulty and in those the response is unsatisfactory. Iron sprays are generally unsatisfactory because of immobilization in the zone of absorption, but in a few species they are the least unsatisfactory method of control of iron deficiency. The relative mobility of molybdenum absorbed by leaves is not yet known, but the responses reported by Stewart & Leonard (23) suggest that it may be classified with manganese.

Although, as we have seen, there are special situations in which foliar applications of the major elements nitrogen and magnesium give satisfactory plant responses, the positive responses to potassium and phosphorus sprays that have been reported are less promising. With all four of these nutrients, the plant requirements are so much greater than the requirements for the trace elements that several sprays at the maximum safe concentrations of the compounds have usually been necessary to bring about the minimal responses that were considered necessary. The number of sprays of these nutrients reported as needed for the response required have ranged from three to more than six. As would be expected, the foliar application of nutrients to plants which already have ample quantities of them do not give additional plant responses that are desirable. This is brought out in the studies of Brasher, Wheatley & Ogle (39).

Economic materials and methods of application.—In addition to the two considerations above, foliar application of nutrients usually cannot furnish a satisfactory alternative to other methods of application unless the costs of the materials applied and the methods of application are competitive with the costs of materials and application by alternative means. If there were no other reason that the proprietary full nutrient spray materials at present on the market were uneconomic, their cost alone would usually rule them out for commercial operations. Given materials at competitive cost, the cost of

application is a matter of primary importance. In general, the capital expense of modern spray machinery cannot be borne by a foliar nutrition program; on the contrary, foliar nutrition of a crop is usually feasible because spray machinery and a spray program exist in the operation anyway.

In view of the complex physiological and economic problems discussed above, it seems unlikely that foliar application of nutrients will ever supplant soil application as a general practice. It also seems unlikely that proprietary mixtures of nutrients for foliar application serve a purpose that is generally useful. On the other hand, it is clear that foliar nutrition is a satisfactory means of dealing with a number of special problems that were not solved by other means. Undoubtedly as time goes on more special uses will be made of this method of fertilizing plants.

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MINERAL NUTRITION OF PHYTOPLANKTON[1,2]

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General Discussion

Many studies of the nutrition of phytoplankton have been initiated in an attempt to understand the ecological conditions suitable for their growth. Simultaneously, the advantages of algae as tools for physiological research, primarily in photosynthesis, were recognized, and many studies of nutrition were designed to provide suitable and reproducible cultures for these purposes. More recently the possibility of the use of organic material from algae as a source of food has stimulated extensive studies of mass cultures.

Because of the different viewpoints of investigators studying the nutrition of unicellular algae a variety of approaches have been used, which makes it difficult to compare the results. It seems worthwhile, therefore, to attempt to define more precisely four ways in which nutrient requirements have been studied and expressed. These are the absolute, the normal, and the minimum requirements for nutrients and their optimum concentrations.

The *absolute requirement* for a nutrient is based upon the postulate that the algae cannot grow, reproduce, or photosynthesize if the nutrient is lacking from the environment, and that no other nutrient can be substituted for it. It is basic to the understanding of the ecology and to the development of culture media since it determines the quality of the environment required. Hutner *et al.* (68) have discussed the value of chelating agents and suggest a rigorous technique for establishing absolute requirements in microbial nutrition.

The *normal requirement* is the quantity of each nutrient contained in cells produced during active growth of a population while no nutrient is limiting. This requirement is based upon the postulate that there is an ideal or normal composition of the cell, and that the algae tend to produce such cells if the environment permits. The normal requirement is as yet, and may always be, ill-defined because of the variability of composition of phytoplankton. However, the cells, by selective absorption, may assimilate a large proportion of an element in limited supply, without assimilating more than a small proportion of a nutrient present in excess. Variations in the composition of the cells thus tend to be less than variations in the composition of the medium. It should be possible to design a balanced medium from which all nutrients are removed at equal rates relative to their concentrations, so that the cells produced would be of the same relative nutrient composition as the

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[1] The survey of the literature pertaining to this review was concluded in August, 1953.

[2] Contribution No. 687 from the Woods Hole Oceanographic Institution.

medium. Such cells could be defined as "normal" cells. The normal requirements for *Chlorella*, as given below, indicate that, for every 100 parts by weight of carbon, about the following amounts of other major nutrients are required: N—15; P—5; Mg—2.5; K—1.8; S—1.6.

The *minimum requirement* is the quantity of a nutrient in the cell when the nutrient is limiting the growth of the population, all other nutrients being present in excess. It determines the mass or number of cells which can be produced for a given quantity of a nutrient, provided light or some other environmental factor does not limit the growth of the population. Cells containing this minimum quantity are deficient cells which would contain a greater amount if it were available. Algae deficient in nitrogen and phosphorus can be produced in this way, and these are capable of assimilating the nutrient from an enriched medium in the dark (59, 71, 106). This technique has been valuable in studying the internal metabolism of these nutrients (28, 86, 119, 120).

The optimum concentration or range of concentrations will permit the maximum rate of growth, reproduction, or photosynthesis of an algal population. It is not certain that the same concentration will be optimal for these three criteria. The optimum concentration is based upon the concept that too low a concentration may be limiting as a result of a decreased rate of assimilation, and that too high a concentration may be inhibitory or toxic. The lower limit may sometimes be confused by the complete removal of the nutrient from the medium as a result of growth of the population. This would be an indication of the minimal requirement rather than of a suboptimal concentration. Ideal studies of optimum concentrations would provide a constant environment by a continuous flow (85) or diffusion system (106) to eliminate the possibility of exhaustion.

The study of these nutritional requirements necessitates the use of pure or, at least, unialgal cultures. All requirements can be determined by cultures and absolute requirements can be established only by cultures. However, very few species have been obtained in pure culture compared to the enormous number of species found in the natural environment, and some species have proved difficult, others impossible to obtain in culture in the present state of our knowledge. Bold (12) lists, with references to original publications, over 200 genera of algae which have been cultured in the laboratory. Of these 82 genera include planktonic algae which are distributed as follows among major groups: 9 Myxophyceae, 39 Chlorophyceae, 6 Euglenaphyceae, 5 Heterokontae, 8 Dinoflagellatae, and 15 Bacillareae.

Most of the investigations of nutrition, however, have been made on the species of algae which are easy to culture. It is obvious that these algae may differ in nutrition and physiology from those which are difficult to culture, and this may be the clue to the difficulty. Presumably, if enough were known concerning their nutrition, these species could also be cultured. In the meantime, evidence from the composition of natural collections and the

changes in the environment which result from their growth may produce clues which will ultimately make their culture and better knowledge of their nutrition possible.

Evidence from the chemical composition of natural populations has the advantage of giving relative proportions in which the nutrients were assimilated under specific conditions of growth. When the conditions of growth are known such analyses are valuable in determining the normal and minimal requirements. If complete, the analysis will include all the nutrients required for growth, but it cannot establish an absolute requirement since the cells may accumulate elements which are unnecessary. Such evidence is, therefore, suggestive of the necessity and proportions but is not conclusive evidence for the requirements.

The changes in nutrient salt concentrations in the environment permits studies of the rates of absorption of nutrients, and will show when the nutrient is approaching limiting concentrations. This type of observation has been made extensively in surveys of natural environments. When the minimum requirement for the population is known the determination of available nutrients may indicate the productive capacity of the environment. Armstrong & Harvey (5) have used the total phosphorus content of the water of the English Channel as a measure of its capacity to produce phytoplankton.

Normal requirements of marine populations have also been determined by the changes of the nutrient salts in nature. It was observed by Harvey (51, 56) and Redfield (104) that both nitrate-nitrogen and phosphate-phosphorus are completely removed from the surface water of the sea at about the same time as a result of the growth of phytoplankton. Redfield correlated the changes in these elements in the sea water and concluded that the ratio $C:N:P$ of removal was $100:16.7:2.5$ gm. Similar average proportions were observed by analysis of collections of plankton. Fleming (33) has collected analyses of plankton and presents a table of "plankton equivalents" which interrelate various physical and chemical properties of phytoplankton communities. For the study of the ecology of plankton production in the sea, these ratios have been of great value, and are comparable to, though much less precise than, the combining proportions of chemistry.

As early as 1896 Molisch (80) stated that the mineral nutrition of the algae was not unlike that of higher plants. This general conclusion has been reaffirmed many times. The major absolute requirements include carbon, nitrogen, phosphorus, sulphur, potassium, and magnesium. Iron and manganese are required in smaller amounts, sodium is not essential, and the evidence for a requirement for calcium is contradictory. Silicon is a major requirement for diatom growth but is unnecessary for other forms. Various other elements such as zinc, boron, cobalt, molybdenum and copper may be necessary as trace elements. These are frequently supplied by adding such solutions as Arnon's (6). In the sea, and in most lakes, all of the major

elements are present in excess with the exception of nitrogen and phosphorus. The specific requirements for these elements will be discussed below.

Many different types of nutrient media have been developed for culturing algae. There is no general agreement concerning a best medium, nor do the media designed for different groups of algae vary more than the media designed by different investigators for the same alga. As Pringsheim (102, p. 32) points out

> Most algae are not affected by minute changes in the composition of the medium, otherwise they could not live under natural conditions. Changes effected by the algae themselves are often more decisive than differences between various media.

In general the media can be divided into two major types, those which are added to enrich natural waters and completely synthetic media made up entirely of purified salts and distilled water. The complete composition can be known only of the second type since the natural waters may add essential nutrients omitted in the enrichment solutions. Some of the media have obviously been designed to simulate natural conditions as closely as possible while others were apparently designed to produce a large population. Table I represents the major constituents of some of the synthetic media. These

TABLE I

CONCENTRATION OF MAJOR NUTRIENTS (MG. PER LITER) IN
VARIOUS CULTURE MEDIA

	Ca	Mg	K	Na	S	N	P	Fe	Mn	Si
Rodhe (106)	14.7	1.0	2.2	7.5	1.3	10.2	0.89	0.18	0.01	4.6
Chu (19)	2.0	2.4	24.0	2.0		5.0	18.0	0.8		1.8
Chu (19)	91.0	7.6	18.7			17.0	9.0			9.1
Tanada (121)	0.3	6.1	399.0	1.8	8.4	87.0	64.0	0.18	24.0	
Gerloff, et al. (41)		0.13	2.25		0.8	13.6	0.45	0.03		
Witsch (125)	12.2	9.8	100.0		13.0	106.0	34.0	3.5		
Scott (110)	244.0	50.0	202.0		66.7	171.0	57.0	4.4	1.0	
Myers & Clark (85)		240.0	820.0		323.3	168.0	279.0	7.0	0.5	
Craig & Trelease (27)		486.0	1680.0	2.0	640.0	350.0	558.0	0.55	0.44	0.06
Geoghegan (39)		486.0	1320.0	2.0	1240.0	305.0	558.0	0.55	0.44	0.06

have been selected solely to indicate the range of concentrations which have been used and not because of their suitability or general use. Rodhe's (106) and Chu's (19) media were designed to simulate natural conditions and the contents of the various salts were adjusted as a result of physiological experiments on, respectively, the green algae *Ankistrodesmus falcatus* and *Pediastrum boryanum* and the diatom *Asterionella gracillima*. Tanada's (121) medium was also used for a diatom, *Navicula minima*, and that of Gerloff, Fitzgerald & Skoog (40, 41) was used successfully for the culture of 22 species of blue green algae. The media of Witsch (125), Scott (110), Myers & Clark (85), and Craig & Trelease (27) have all been used for the

mass culture of *Chlorella* and are arranged in order of increasing total molarity which ranges from 6 to 64 millimols per liter. Geoghegan's (39) medium is derived from that of Craig and Trelease, but uses ammonium rather than nitrate-nitrogen. These media provide a wide range of total concentration and of each individual salt. The ratio of nitrogen to phosphorus, for example, in the various media used for *Chlorella* varies five-fold. The fact that luxurious growth of *Chlorella* is possible in these media emphasizes its marked adaptability to variations in environmental conditions.

Some algae which will not grow on completely synthetic media will grow when soil extracts, yeast extracts or other complicated mixtures are added. Sometimes the use of natural water in place of distilled water renders a medium suitable. Pringsheim has used soil and peat extracts extensively and effectively in obtaining growth of cultures and has recently (102) reviewed their value. He concluded that these may act through providing trace elements or organic growth factors or because the humic acids may form complexes with essential elements keeping them present in an available form.

The use of chelating compounds in culture media has been introduced by Hutner *et al.* (68). These compounds form complexes with trace elements and thus prevent their precipitation. Enough ions are released through mass action to satisfy the requirements of the growing cells. Ethylenediaminetetraäcetic acid (EDTA) is the best known of the chelating agents, and Schwarzenbach & Freitag (109) have determined equilibrium constants for a number of metals and EDTA in unimetallic solution. Since many required elements may be toxic in high concentrations, chelating agents may permit the growth of an organism in a medium containing a quantity of a trace element which would otherwise be lethal.

ESSENTIAL NUTRIENTS

Carbon.—Many of the algae included among the phytoplankton have heterotrophic, chemotrophic, and autotrophic types of metabolism, and some species may exhibit all three under appropriate conditions. Fogg (35) has recently reviewed the chemotrophic assimilation of carbon. The use of organic carbon sources has been discussed by Doyle (29), Hutner & Provasoli (67), Algeus (1), Barker (11), Bristol-Roach (16, 17), Myers (84), and Pearsall & Bengry (94). Radioactive carbon has been valuable in studies of carbon assimilation and the products of photosynthesis [Calvin *et al.* (18); Gaffron *et al.* (37, 38)], and Steemann Nielsen (116) has proposed a method for determining the productivity of natural waters from the rate of assimilation of radioactive carbon. The autotrophic, or photosynthetic, assimilation of carbon will be considered here, since it is this process which makes the phytoplankton important producers of organic material in their natural habitat.

The normal requirement for carbon of various algae grown with continuous illumination and adequate nutrients varies from about 51 to 56 per cent

of the ash-free dry weight (73). Spoehr & Milner (113) have shown that the carbon content of *Chlorella pyrenoidosa* may be varied from 49.5 to 70.17 per cent of the ash-free weight by modification of the medium. The highest carbon contents, associated with storage of fats, were obtained in cultures grown in continuous light, with a low concentration of fixed nitrogen, and provided with a gas mixture of 5 per cent CO_2 in nitrogen.

The quantity of carbon dioxide, bicarbonate and carbonate ions present in the water is a function of temperature, pH, excess base, and the carbon dioxide content of the gas with which the water is in equilibrium. Harvey (56) discusses the various equilibria involved and the means for computing the total carbonate and free CO_2 in sea water. The free CO_2 in the medium decreases from 100 per cent at pH 4 to virtually zero concentration at 9. The carbonate concentration increases from zero at a pH of about 7.5 to 100 per cent at pH 12. The bicarbonate is at a maximum at intermediate pH values. As a result of this complicated equilibrium, the evaluation of the utilization of the different ions has been difficult to separate from the direct effect of pH.

It has been generally accepted that the algae use free CO_2 in photosynthesis since Warburg (124) showed in 1919 that the rate of photosynthesis of *Chlorella* was dependent on the concentration of undissociated CO_2, even though both ionic forms were present in excess. Osterlind (91, 92) showed that *Scenedesmus quadricauda* can also utilize bicarbonate ions although *Chlorella pyrenoidosa* can not. Steemann Nielsen (114, 115) shows that bicarbonate ions can be utilized by other aquatic plants. Apparently carbonate ions cannot serve directly as a source of carbon in photosynthesis and Osterlind (90) indicates that an excess may inhibit growth.

It has been shown that *Chlorella* and *Hormidium* are photosynthesis-saturated at about .03 per cent carbon dioxide [Emerson & Green (30); Honert (60)] and that *Scenedesmus* is growth-saturated at this level [Osterlind (91)]. Myers, (81) has shown, however, that if the rate of carbon dioxide assimilation by the cells is high it becomes physically impossible to maintain this level without bubbling a carbon dioxide mixture through the culture. Concentrations of 1 to 5 per cent are generally used. Spoehr & Milner (113) showed that dense cultures of *Chlorella pyrenoidosa* which depended on air alone for their source of carbon produced only one tenth the weight of cells produced under otherwise similar conditions by cultures which received 5 per cent CO_2 in air. The carbon dioxide was also provided in nitrogen instead of air, at concentrations of 3, 5, and 10 per cent. The latter inhibited growth and the 3 per cent concentration limited growth with high light intensities, but gave yields equal to the 5 per cent concentration at low light intensities.

It is probably rare that the production of plant material in aquatic environments is limited by the supply of inorganic carbon since the total carbonate is generally present in great excess. The ability of some organisms

to utilize the bicarbonate ion, which is at maximum concentrations at about pH 8, may be important in the success of some forms, especially in the open sea where the pH is generally uniform at about this value.

Nitrogen.—The normal requirement for nitrogen in cultures of various Chlorophyceae was found by Ketchum & Redfield (73) to be about 6.5 to 8.3 per cent of the ash-free dry weight. *Chlorella* cultures, in which the amount of growth is determined by light intensity, contain similar proportions of nitrogen in the cells [Myers (82)]. There is considerable variation, however, since cells which are deficient or which exhibit a luxury consumption of nitrogen, can be produced by changes in culture conditions (71, 73, 74, 113).

Nitrogen-deficient cells are produced if cultures are allowed to grow in the light after all of the nitrogen has been assimilated from the medium. The nitrogen content of these is about one-third (2 per cent of ash-free dry weight) of that in normal cells (71, 73). Spoehr & Milner (113) found that *Chlorella* with large fat reserves could be produced in a low nitrogen medium provided with 5 per cent CO_2 in nitrogen gas and continuous illumination at moderate intensity. Such cells contained as little as 1.17 per cent nitrogen on an ash-free basis. Nitrogen-deficient cells assimilate nitrogen from nitrate or ammonia in the dark, whereas normal cells are unable to do so without an organic carbon supply. Harvey (59) has shown that nitrogen-deficient *Nitzschia closterium* are unable to assimilate nitrite nitrogen in the dark.

A luxury consumption of nitrogen has been described by Kraus (74) who found that *Scenedesmus obliquus*, containing more than twice the normal nitrogen, were produced if growth was limited by a deficiency of the trace elements manganese, boron, and zinc.

Nitrogen-deficient *Chlorella* exhibit a complete carbohydrate metabolism for short periods, as shown by Myers & Cramer (86) and Spoehr & Milner (113) show that in older cultures nitrogen deficiency results in fat storage. The respiration rate of nitrogen-deficient cells is low [Syrett (119)] but increases as soon as ammonium sulphate is added. During the assimilation of ammonium nitrogen the respiratory quotient was unusually low (28, 119), though Cramer & Myers (28) observed high respiratory quotients when nitrate and glucose were supplied to nitrogen-deficient *Chlorella* in the dark. Syrett & Fowden (120) showed that the first products of the dark assimilation of ammonium nitrogen were principally amide and free amino acid nitrogen. Without added glucose much of the assimilated nitrogen remained in this form, but when glucose was added the assimilated nitrogen was incorporated into insoluble protein fractions. Harvey (59) also showed that the assimilation of ammonium or nitrate nitrogen by deficient *Nitzschia closterium* produced mainly nitrogen compounds soluble in hot water, and also that the chlorophyll content of the cells was greatly increased during recovery from nitrogen deficiency in the dark.

Nitrogen in the fixed form is generally available in aquatic environments

as nitrate, nitrite, and ammonia. In the open ocean the concentration of nitrate nitrogen reaches a maximum of about 0.6 mg. per liter and this is about ten times as great as the maximum for the other two inorganic forms (118). Chu (19) summarizes the composition of various fresh waters and presents average nitrogen concentrations in surface waters ranging from 0.31 to 8.7 mg. per liter. As a result of growth of the phytoplankton, the fixed nitrogen in surface waters may be reduced to unmeasurable concentrations [Harvey (51); Redfield (104)]. Marine animals excrete nitrogen largely as ammonia, urea, uric acid, trimethylamine oxide and amino acids [Harvey (56)] and polluted waters may contain various organic forms of nitrogen. Cooper (26) has reviewed the forms in which compounds of nitrogen may be expected in the sea. The ability of phytoplankton to use these different forms of nitrogen directly may be of ecological importance for their survival.

Nitrogen fixation has been demonstrated for various Myxophyceae, and the evidence has been reviewed by Fogg (34, 35). There is no satisfactory evidence that algae other than Myxophyceae are capable of fixing nitrogen, though Spoehr & Milner (113) reported that the cellular nitrogen content of some of their *Chlorella pyrenoidosa* cultures was substantially more than the fixed nitrogen supplied in the medium. They do not consider their results conclusive. It is possible that other forms capable of fixing atmospheric nitrogen will be found, since very few have been investigated critically for this property.

Many investigations have shown that both ammonia and nitrate are readily available sources of nitrogen in algal cultures (28, 95, 96, 101). The availability of the ammonium and nitrate ions is related to the pH of the medium and, conversely, their assimilation changes the pH. The assimilation of ammonium ion by *Chlorella* can reduce the pH of the medium to values as low as pH 3.0, whereas assimilation of nitrate can increase the pH to 7.0 from initial values of pH 4.5 [Pratt & Fong (101)]. In growing mass cultures of *Chlorella vulgaris*, Geoghegan (39) found better yields with ammoniacal nitrogen when the pH of the medium was adjusted to 6.0 twice daily and never allowed to fall below 5.0.

The availability of these two forms of nitrogen may be different for different algae or in different culture media. Ammonium nitrogen was more rapidly assimilated than nitrate nitrogen from the same solution by cultures of the marine diatom *Nitzschia closterium* [ZoBell (126)] and by mixed phytoplankton populations [Harvey (55)]. In the latter experiments, practically all of the ammonium nitrogen was assimilated before there was any appreciable utilization of nitrate nitrogen, even though the latter was 60 times more concentrated in the enriched sea water. Ryther (107) found, however, that *Nitzschia closterium* was unable to grow with ammonium nitrogen, though *Nannochloris* and *Stichococcus* grew more rapidly on ammonium than on nitrate nitrogen.

Nitrite is present in natural waters at low concentrations and is an

available source of nitrogen. Ludwig (76) found it toxic to *Chlorella* in higher concentrations, and ZoBell (126) found it less toxic than ammonium but more toxic than nitrate nitrogen in his sea water cultures of *Nitzschia closterium*.

The availability of other forms of nitrogen has also been investigated. Ludwig (76) studied the ability of *Chlorella* to use many forms of nitrogen, and his bibliography of over a hundred references is an excellent review of earlier work. Acetamide, urea, guanidine carbonate, uric acid, several amino acids, and peptone were suitable sources of nitrogen. Schreiber (108) found that bacteria-free cultures of *Carteria*, a marine flagellate, can utilize glycine, and Braarud & Føyn (15) found that *Chlamydomonas* can utilize glycine, alanine, and asparagine. Algeus (1, 2, 3, 4) has investigated various organic nitrogenous compounds in the nutrition of several Chlorophyceae. In some the rate of nitrogen assimilation was limited by deamination; in others, with more rapid deamination, ammonia was excreted into the medium.

The optimum concentration of the nitrogen supply is apparently very different for different species. *Chlorella* is very tolerant of variations in nutrient concentrations, and Myers (83) found that the major salts could be varied by dilution of the entire medium twenty-fold without influencing growth or photosynthetic behavior. The composition of his undiluted medium is given in Table I and contains 168 mg. of nitrogen per liter. Ammonium nitrogen concentrations greater than 31.5 mg. per liter were found by Spoehr & Milner (113) to inhibit the growth of *Chlorella pyrenoidosa*, though Geoghegan (39) found that *Chorella vulgaris* grew well at much higher ammonium nitrogen concentrations provided the pH of the medium was controlled.

The marine diatom *Nitzschia closterium* also appears to grow well regardless of variation in nitrogen concentration. The rate of growth of cultures of *Nitzschia closterium* was independent of the nitrate nitrogen concentrations between 0.05 and 0.5 mg. per liter [Ketchum (70)], though the rate of assimilation of nitrogen was decreased when the medium contained less than 0.2 mg. per liter. Under different culture conditions Ketchum & Redfield (72) obtained about the same rate of growth with this marine diatom in a medium which contained 15 mg. N per liter. ZoBell (126) found that the upper limits of concentration for optimum growth of *Nitzschia closterium* in ammonium, nitrite, and nitrate-nitrogen media were respectively 0.7, 70, and 560 mg. N per liter.

Chu (20) found, however, that the rate of growth of various species was limited by concentrations greater than 5 to 17 mg. N per liter as shown in Table II. These concentrations are much less than those commonly used in culture media for *Chlorella* (cf. Table I), but are greater than those normally found in nature. Little difference was found between ammonium and nitrate nitrogen in Chu's experiments. Rodhe (106) found that the initial rate of growth of *Ankistrodesmus falcatus* was equal for nitrogen concentrations from

0.15 to 10.0 mg. N per liter whether the nitrogen was supplied as ammonium chloride or calcium nitrate. After longer times, however, growth was limited in the lower concentrations presumably because of the complete removal of nitrogen from the medium. The growth rate also decreased in the higher ammoniacal nitrogen concentrations, being less in the 10 than in the 5 mg. N per liter after six or more days. He attributes this inhibitory effect to the rapid assimilation of CO_2 by highly productive cultures with resultant pH values of 11 or higher, thus liberating free ammonium hydroxide in the culture.

Dinoflagellates can utilize very low concentrations of nitrogen, and Barker (10) found that growth rate was not limited unless the sodium nitrate

TABLE II

MAXIMUM CONCENTRATIONS (MG. N PER LITER) OF NITROGEN FOR OPTIMUM
GROWTH OF VARIOUS ALGAE. [AFTER CHU (20)]

Species	$NO_3^- - N$	$NH_4^+ - N$
Pediastrum boryanum	10.4	10.6
Staurastrum paradoxum	8.5	5.3
Botryococcus Braunii	6.9	
Fragillaria crotonensis		13.1
Nitzschia palea		6.5
Asterionella gracillima	17.1	

enrichment was less than 0.001 mg. per liter. Additions of more than 0.01 mg. ammonium chloride per liter inhibited growth of *Prorocentrum micans* and a *Peridinium*. Gerloff, Fitzgerald & Skoog (41) found that the yield of cultures of the blue-green alga *Coccochloris peniocystis* increased with increasing nitrate content of the medium up to a concentration of 13.6 mg. N per liter.

It appears, therefore, that *Chlorella* and *Nitzschia* are relatively insensitive to variations in the nitrogen content of the medium (provided it is present in excess) but that some species of algae can be inhibited in rate of growth by excessive nitrogen concentrations. Additional studies would be desirable especially concerning the effect of pH on optimum nitrogen concentrations. These should be conducted under continuous flow or diffusion conditions to avoid exhaustion of the nutrient medium (85, 106).

Phosphorus.—The normal phosphorus requirement of various Chlorophyceae is about 2 to 3 per cent of the dry weight of cells (73). This a greater proportion than Redfield (104) found for natural collections, though marine diatoms, having heavy siliceous shells would be expected to contain somewhat less phosphorus on the dry weight basis (33, 73). The phosphorus content can also be varied greatly by rearing cultures in deficient media

(71, 73), and luxury consumption with storage of excess phosphorus has been observed by Lund (77), Goldberg *et al.* (43), and Rodhe (106).

Phosphorus-deficient cells can be produced by permitting cultures to grow and reproduce after complete removal of all of the phosphate from the medium. Franzew (36) produced deficient *Scenedesmus quadricauda* and *Pandorina morum* in this way which contained as little as one-ninth the normal phosphorus content. Deficient cells of *Nitzschia closterium* and *Chlorella pyrenoidosa* were produced in the same way by Ketchum (71) who showed that such cells were capable of assimilating phosphate from the medium in the dark to satisfy the phosphorus debt thus incurred, though cells grown in a complete medium did not assimilate phosphate in the dark. Phosphorus-deficient *Chlorella pyrenoidosa* assimilates potassium as well as phosphorus in the dark, the two elements being absorbed in approximately equivalent amounts on a molar basis [Scott, (112)]. Calcium and magnesium were not assimilated under these conditions.

Rodhe (106) indicates that, in addition to satisfying the phosphate debt by assimilation in the dark, *Scenedesmus quadricauda* can store excess phosphorus provided adequate supplies are available. Such cells, when returned to the light in a phosphorus-free medium were able to grow as rapidly as those in an adequate medium. The assimilation to satisfy the phosphate debt proceeded very rapidly (one day), but the accumulation of extra phosphorus required a fairly long time (7 days).

The content of phosphorus in the cells can be expected to be variable, especially when grown in media containing low concentrations of phosphate. It was shown, for example, by Ketchum (70) that the rate of assimilation of phosphorus by *Nitzschia closterium* was directly related to the phosphorus concentration below about 0.1 mg. P per liter and also to the concentration of nitrate nitrogen below 0.2 mg. N per liter. In these experiments phosphorus concentrations less than 0.017 mg. P per liter limited cell division as well as the rate of assimilation. Lund (77) has shown that *Asterionella formosa* is variable in the amount of phosphorus contained per cell, and he attributes the difference to storage of excess phosphorus by cells grown in phosphate-rich media.

Goldberg, *et al.* (43) using radioactive phosphorus found a linear relationship between the phosphorus content of the diatom *Asterionella japonica* during exponential growth and that of the medium within the range .015 to .110 mg. P per liter. The range of variation within the cells was, however, only about one-half of the range in the medium. As much as 50 per cent of the radioactive phosphorus in cells containing more than the minimum requirement could be removed by washing with sea water free of radioactive phosphorus [Goldberg *et al.* (43)]. The minimal amount of phosphorus was found to be strongly bound, since little regeneration in the medium was observed after three weeks storage in the dark, although the cultures were not bacteria free.

The physiological state of the cells may also be important in determining their phosphorus content. Old dense cultures of *Stichococcus bacillaris* were found by Ketchum & Redfield (73) to have high phosphorus content, though *Chlorella pyrenoidosa* showed no progressive change of phosphorus content with age of the culture.

Without analysis of the cells, or experiments to determine whether phosphorus assimilation can proceed in the dark, it is impossible to differentiate between excess storage, normal content, and deficient cells.

Ortho-phosphate is the form normally found in natural waters and used in culture media. Chu (22) reports that pyro-phosphate cannot be used by *Phaeocystis pouchetti*, *Skeletonema costatum*, and *Nitzschia closterium* as effectively as the ortho-phosphate. Bacteria-free cultures of *Nitzschia closterium* can utilize inositol hexaphosphate and glycerophosphate when growing in the light [Chu (21)], and Harvey (58) showed that phosphorus-deficient cells can also assimilate these compounds in the dark.

The optimum phosphorus concentrations for growth of the various species listed in Table II was studied by Chu (20). The upper limit which permitted maximum growth rates varied from 8.9 to 17.8 mg. P per liter, higher concentrations being inhibitory. Gerloff, Fitzgerald & Skoog (41) obtained maximum rates of growth with the blue-green *Coccochloris peniocystis* at phosphorus concentrations of 0.45 mg. P per liter. Their highest concentration tested contained 5.4 mg. P per liter and was not inhibitory. Rodhe (106) found very different optimum requirements for various species. *Ankistrodesmus falcatus* reproduced at the same rate for the first three days in cultures containing 0.005 to 1.0 mg. P per liter. With increasing length of time the growth rate decreased in progressively higher concentrations so that after eight days maximum growth was found only in the highest concentration. For the lower concentrations (<0.2 mg. per liter) the number of cells produced after 15 days was directly proportional to the concentration of phosphorus in the medium, indicating that the minimum phosphorus requirement was limiting growth. In higher concentrations the direct relationship was not found and Rodhe concluded that, in these cultures, the nitrogen supply was limiting. The growth of *Scenedesmus quadricauda* also increased as the phosphorus concentration increased up to 1.0 mg. per liter, but additions of only 0.002 mg. P per liter to Lake Erken water gave maximum growth of *Asterionella formosa* and addition of 0.05 or more mg. per liter inhibited growth of this form. Preliminary observations indicated that additions to Lake Erken water of 0.005 or more mg. P per liter was sufficient to inhibit growth of *Dinobryon* and *Uroglena*. It is of interest that *Dinobryon* blooms occur in lakes after a decline of other species. Pearsall (93) correlated these blooms partly with nitrogen:phosphorus ratios, a high ratio being favorable. Chu (22) however, concludes that this ratio is unimportant while each element remains within the optimum range of concentrations. Hutchinson (69) found evidence that *Dinobryon* can increase at lower nutrient con-

centrations than antecedent species which might be diatoms, *Scenedesmus* or blue-green algae. The inhibition of growth by relatively low concentrations of phosphorus may be the explanation for the failure of *Dinobryon* to bloom except following growth and the resultant assimilation of nutrients by other species.

Sulfur.—Kraus (74) reports a sulfur content of *Scenedesmus obliquus* of 0.91 per cent of the total dry weight. Sulfur is an invariable constituent of living matter, and is generally provided as sulfate in culture media. However, no studies on the effects of varying concentrations of sulfate on the growth of alage have been found. Harvey (56) reports that the normal sulfate content of sea water suffices for the growth of a number of planktonic diatoms.

Harvey (54) found that the marine diatom *Ditylum* was unable to grow normally in some samples of sea water collected during summer months unless a compound containing divalent sulfur was added. Sodium sulfide or the organic compounds cystine, glutathione, methionine, and thiamin were found to restore the fertility of the water. Matudaira (79) found that additions of 0.1 to 1.0 mg. per liter of $Na_2S \cdot 9H_2O$ stimulated the growth of *Skeletonema costatum* in sea water, but that 5 mg. per liter arrested cell division and no cell division occurred when 10 mg. per liter was added. Cystine and alanine were also effective. Auxospore formation was promoted by the addition of sulfides. Since he was unable to grow this diatom in artificial sea water, with or without added sulfide, he concluded some unknown accessory was also needed.

Calcium and magnesium.—These two elements are discussed together because studies have generally been made by substituting a salt of one for the other in the culture medium.

It has frequently been shown that calcium is not essential for the growth of *Chlorella* (63, 81, 110, 123) and many culture media (cf. Table I) omit calcium. Rodhe (106) found no effect on growth of *Ankistrodesmus falcatus* as a result of varying the calcium content from 1 to 10 mg. calcium per liter, and Trelease & Selsam (123) found no effect on growth of *Chlorella* by additions up to 160 mg. per liter. Gerloff, *et al.* (41) found calcium unnecessary for the growth of the blue-green *Coccochloris peniocystis*. Manuel (78) and Noack: *et al.* (88) found, however, a need for calcium by *Chlorella*, and Hutner (66) has found a calcium requirement of 5 mg. per liter for the marine diatom *Nitzschia closterium*. Chu (22) indicates that the minimum value of calcium for good growth depends upon the medium used and upon the species of algae. It is possible that some of his results reflect a pH effect which is not recorded.

The absolute requirement for magnesium is well established. The addition of as little as 0.1 mg. per liter produced optimum growth of *Ankistrodesmus falcatus,* and no inhibition of growth was found up to 10 mg. per liter [Rodhe (106)]. Different optimum concentrations for various species and for different media were reported by Chu (22). The minimum concentration which

produced good growth in his medium 9 was 0.01 mg. per liter for diatoms, though 4.0 mg. per liter was required by *Staurastrum paradoxum*. Concentrations which inhibited growth in this medium ranged from 48 mg. per liter for diatoms to 1 mg. per liter for *Pediastrum boryanum*. Trelease & Selsam (123) found that the concentration had to exceed 480 mg. per liter to inhibit growth of *Chlorella*. Some of the discrepancies may not be due to different species or media, since Finkle & Appleman (32) have shown that concentrations less than 2.8 mg. per liter reduced the division rate of *Chlorella*, but not the rate of production of dry weight.

Hutner (66) has used a "citrate extinction" technique to determine both the calcium and magnesium requirement of the marine diatom, *Nitzschia closterium* in a completely artificial medium. The method depends upon the determination of magnesium or calcium requirement at various citrate concentrations and extrapolation of the results to zero citrate where the total concentration would be present in the ionic form. The minimum concentrations obtained in this way were about 5 mg. Ca, and 40 mg. Mg per liter.

The calcium and magnesium content of *Chlorella pyrenoidosa* when grown in media containing various proportions of these two elements has been determined by Scott (110). No inhibition of growth was found when calcium was omitted, and the cells produced contained no calcium, indicating that no appreciable supply was available as a contaminant. The ratio of calcium to magnesium in the cells gave a linear relationship with the ratio in the medium, though a three-fold variation in the medium produced only a 1.4 fold variation within the cells. In these experiments the calcium content, on an ash-free dry weight basis, varied from 0 to 0.58 per cent and the magnesium content varied from 0.29 to 1.37 per cent. Scott (111) further found that the calcium content was replaced to a large extent by magnesium and partially replaced by sodium or potassium when the cells were washed in distilled water solutions of the chlorides of these ions. Part of the magnesium, however, was firmly bound and this quantity was greater than the magnesium present in the chlorophyll.

Sodium and potassium.—There is no evidence for a requirement of sodium for algae. Pratt (100) has observed an effect of the sodium:potassium balance on photosynthesis of *Chlorella* in bicarbonate buffers, and Emerson & Lewis (31) have reported a high sodium, low potassium requirement for *Chroococcus*. Media containing no sodium, however, permit an abundant growth of a large variety of algae.

The effects of potassium have been extensively studied by Pirson (97, 98) and Pirson & Wilhelmi (99). Potassium-deficient cells show a low rate of growth and photosynthesis, and a high rate of respiration. Rapid restoration to normal was observed following the addition of potassium or rubidium, but lithium, sodium, and cesium were not effective. Scott (110) found the growth of *Chlorella pyrenoidosa* was depressed with less than 7.8 mg. K

per liter in the medium and both the potassium and phosphorus content of
these cells was less than normal. Increasing the potassium to double this con-
centration, however, resulted in no further uptake of this ion and these
cells contained about one per cent potassium. At low concentrations of po-
tassium, the sodium content of the cells was considerably increased, but at
higher concentrations it remained constant at about 10 per cent of the
potassium in the cells. Provided sufficient potassium was available, the ratio
of potassium to sodium within the cell was independent of the ratio in the
medium. The potassium was firmly bound within the cells and could not be
removed by washing them with distilled water solutions of sodium, potas-
sium, or magnesium chlorides [Scott (111)].

   Iron.—The requirement for iron for the growth of algae is well substanti-
ated (61 to 65; 84, 106), though the way in which the iron should be provided
has been a subject of controversy. The solubility of iron in alkaline solutions
is very low and Cooper (25) concluded that less than 10^{-7} mg. per liter of
ionic iron can exist in the sea in equilibrium with ferric hydroxide. Harvey
(53) concluded that the phytoplankton of the sea can utilize iron in colloidal
suspension.

   Goldberg (42) using radioactive iron, found that the marine diatom
Asterionella japonica utilized only particulate or colloidal iron for growth
whereas iron present as a complex of citrate or a synthetic humic acid was
not available. He concluded that a direct study of particulate iron and its
size distribution is necessary in determining the nutrient availability of the
iron. In culture media iron is frequently supplied in an inorganic form, most
of which must be present as colloidal particles in alkaline solutions. Myers
(84) and Gerloff, Fitzgerald & Skoog (41) show that aged nutrient media
containing inorganic iron are less effective than media freshly prepared.
This change must indicate the aggregation of colloidal particles.

   It was suggested by Gran (44) that the early growth of phytoplankton
populations in inshore water and over banks might be due to the presence
of high iron concentrations washed out with humus compounds from the
soil. It is possible that some of the success of soil extract in obtaining cultures
can be attributed to the ability of humic acids to maintain the iron in an
available form [Pringsheim (102)].

   The use of iron as the citrate complex was suggested by Hopkins &
Wann (64), and Rodhe (106) discusses the change in availability of iron in
this form as a result of aging, autoclaving, and light. The citrate complex
was found to be more effective as a source of iron in low concentrations than
ferric chloride but both were equally effective in higher concentrations
[Gerloff *et al.* (41)]. The growth of *Ankistrodesmus falcatus* was negligible
when no iron was added [Rodhe (106)], but 0.04 mg. Fe per liter was suffi-
cient to give maximum growth. Above concentrations of 2 mg. Fe per liter
inhibition of growth was observed. Myers (83) found that concentrations of
iron ranging from 0.01 to 7.4 mg. per liter gave adequate growth of *Chlorella*,

but in continuous flow cultures a concentration of 0.5 mg. per liter limited growth while concentrations above 5.0 mg. per liter were adequate (85).

Silicon.—The growth of diatoms rapidly removes silicates from the surface waters of the sea [Atkins (7, 8); Cooper (24); Hart (49, 50)]. The direct relationship between silicate concentration and diatom growth needs further investigation, though Harvey (52) has shown that addition of silicates to sea water may stimulate growth of *Nitzschia closterium*, and Chu (19) has investigated its requirement by various fresh water diatoms. Harder & Witsch (48) obtained increased yields of moss cultures of diatoms by the addition of sodium "wosserglas" up to 0.16 per cent, though 0.3 per cent produced an inhibition. Precise experiments are needed, however, in which all contact with glass containers is eliminated.

Hart (49, 50) and Clowes (23) believe that low silicate concentrations limit the rate of diatom growth in the antarctic during the summer when unusually thin-walled diatoms were observed. Atkins (9) has compared the changes in silicate and in phosphate content of the waters of the English Channel, and concluded that the large proportion of phosphorus removed indicates that forms other than diatoms must account for a large part of the organic matter production.

Manganese.—Variable and small quantities of manganese have been found in the surface waters of the Pacific by Thompson & Wilson (122). Harvey (54, 57) has shown that the addition of one part of Mn per thousand million parts of sea water is sufficient to produce vigorous growth of *Ditylum brightwelli*, a *Chlamydomonad*, a marine *Chlorella*, a *Cryptomonad*, and two *Chrysomonads*. The addition of manganese to deficient *Chlorella* cells was shown by Pirson (97) to cause an immediate increase in photosynthesis, though Harvey (57) shows a prolonged lag period before the start of active growth of the marine *Chlorella*.

Manganese is important in nitrogen metabolism, and Kylin (75) shows that it stimulated growth of sporelings of *Ulva lactuca* when nitrate, but not when ammonium nitrogen was supplied. Harvey (57) and Noack & Pirson (87) found stimulation with both forms of nitrogen.

Trace elements.—Trace element solutions, such as Arnon's (6), are commonly added to supply possible additional needs of algae. A requirement for traces of zinc has been shown by Ondratschek (89) for various flagellates and by Stegmann (117) for *Chlorella*. Copper may be required [Gusserva (46, 47)] but Greenfield (45) has shown that it inhibits photosynthesis at very low concentrations (10^{-7} M). Provasoli & Pintner (103) have shown that traces of Zn, Mn, Cu and Co are required by several algal flagellates. Gallium was found by Riley (105) to stimulate cell production in cultures deficient in phosphorus and nitrogen, but had no effect if these nutrients were also added. A requirement for molybdenum has been demonstrated for some nitrogen-fixing, blue-green algae [Fogg (35); Bortels (13, 14)].

The establishment of requirements for trace elements demands rigorous

purification of all of the ingredients of the culture medium. As suggested by Hutner *et al.* (68) further improvements in techniques are likely to establish additional requirements which are now unknown because the nutrients are added in adequate supply as contaminants with the various chemicals or water used. The potential value of complexing agents, such as ethylenediaminetetraacetic acid, in establishing requirements for trace elements is stressed by Hutner *et al.* (68) and by Provasoli & Pintner (103).

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SOIL CONDITIONERS[1]

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The advent of synthetic soil conditioners heralds a new phase in agricultural science and in the study of soil structure as a function of organic matter. It is perhaps too early yet to assess the true value of synthetic soil conditioners in agricultural practice, for the first results were only disclosed at a conference at the end of 1951 and published in June 1952. Yet the interest in these new agricultural chemicals has been made so manifest by the many popular articles concerning them, and by the marketing of a great variety of products purporting to act as soil conditioners, that a review of the subject at this early stage seems desirable. The reviewer realizes, however, that with the close of the growing season of 1953 the results of numerous experiments, at present under way, will shortly be published and the present review may well be considered inadequate by the time it appears in print. For this reason, the reviewer will discuss the subject in general, with attention to the historical aspect and with reference to results which appeared before September, 1953.

ORGANIC MATTER AND SOIL STRUCTURE

The importance of organic matter in determining soil structure and, therefore, soil productivity is now well recognized; it has indeed taken a place equal in importance to that of the nutrients as a major factor contributing to soil fertility. The continued supply of organic matter for the maintenance of a good soil structure has become a critical matter in those highly populated areas where intensive cultivation of available agricultural land is essential, or in those areas where erosion, due to adverse climatic conditions, plays a devastating role. Yet in such areas "natural" organic matter may not be available owing to lack of animal manures, or of suitable materials for the preparation of composts or manure substitutes.

It became clear to the reviewer, as Director of the Agricultural Research Council Unit of Soil Metabolism in England during World War II, and engaged in problems concerning the improvement of soil productivity, that the only satisfactory manner of obtaining organic matter which would have the desired effect of improving soil structure, which would be reasonably constant in chemical composition, and which would be freely available under all circumstances, would be to manufacture it from some source of readily obtainable raw material. A closer study of the chemical and physical properties of "natural" soil conditioners thus became very desirable; it became an important aspect of the work of the Agricultural Research Council Unit of Soil Metabolism from 1941 to 1945.

[1] The survey of the literature pertaining to this review was concluded in October, 1953.

Natural Soil Conditioners

Humus.—The most obvious and well-known natural soil conditioner is the complex mixture of substances classed as humus. This was once thought to be formed solely from cellulose but it is now known to be produced partly from the products of cellulose breakdown by microorganisms, partly from fungal mycelia, and partly by chemical transformations of lignin. Humic acid from soil is reported to contain about 5 per cent nitrogen and cannot, therefore, be wholly a lignin or cellulose product. Humus is a natural complex of substances, whose constituents undergo, in soil, continuous change involving syntheses and decompositions. It has, therefore, a variable composition and is composed of derivatives of lignin, protein, hemicelluloses, and cellulose. It consists largely, according to Waksman & Iyer (1), of two groups of substances: (*a*) the lignoproteins combined with calcium, iron, or aluminium ions; these probably combined with the hemicelluloses or polyuronides, form the fraction known as humic acid and are partly responsible for the characteristic color and for some of the colloidal properties of soils; and (*b*) the celluloses, hemicelluloses, waxes, fats, and starches in various amounts and proportions according to the location.

There is now an extensive literature on the formation of soil organic matter by various cropping systems and incorporation of crop residues, and its bearing on soil structure and soil fertility. It is not feasible to summarize such literature in this article but a few recent contributions may be noted. Bruin (2), discussing the content of organic matter in Dutch soils, points out that this may range from a few per cent to over 30 per cent on arable land, and 60 per cent or more in permanent grassland depending on ground water and drainage conditions. Grass yields are highest in soils with 12 to 16 per cent organic matter, higher quantities being accompanied by insufficient drainage and lower quantities by too little moisture supply. Varying aggregation percentages occur with the same content of organic matter, and only very few of the soils show a good structure. There is a good correlation between soil structure and potato yields particularly with optimum N dressings, which are low with a satisfactory soil structure.

The contribution to soil organic matter by cereal residues and undersown crop has been investigated by Smith & Mabbitt (3). They find that the total organic matter in the form of residues supplied by a cereal harvest, undersown with grass or clover, amounts to approximately that supplied by 10 tons of farmyard manure. Page & Willard (4) have shown that a combination of a deep legume and a grass is highly effective in securing an improved soil aggregation. There is a positive correlation between degree of soil aggregation and corn yield [cf. Van Bavel & Schaller (5)].

The decomposition of green fodder, straw, spruce needles, beech leaves, and peat during lengthy periods (4 and 8 years) and under a variety of conditions has been studied by Springer & Lehner (6). They show that the order of decomposition is such that green fodder breaks down faster than straw, and

straw faster than leaves or peat. After four years, the decomposition of cellulose in straw and green fodder was found to be 97 per cent, in leaves about 92 per cent, in needles 85 per cent, and in the peats 54 and 53 per cent. Under anaerobic conditions, a lower rate of breakdown, in the same order, is observed. The largest quantities of humus substances are formed under aerobic conditions after one or two years, the peats being the most productive.

Soil Aggregation

The structure of a soil is largely determined by its crumb or aggregate formation. This greatly influences water movement, aeration, and heat transfer. Many of the biochemical and chemical changes that affect soil fertility take place at the large surface areas presented by soil aggregates. Substances that stabilize crumbs, or aid in soil aggregation, are uniformly distributed in the crumbs of soil of good structure. They consist, according to Kroth & Page (7), of polar organic molecules resulting from the decomposition of fresh organic matter, and of a group of substances including iron and aluminium oxides, fats, waxes, and resins. The latter group is less efficient than the former as soil aggregators. Bacterial slimes and fungal mycelia, it is well known, play an important role in soil aggregation and in increasing the resistance of soils to erosion (8, 9). Among the most important natural soil aggregators are polyuronides, polysaccharides, and cellulose breakdown products.

Polyuronides, Polysaccharides, and Related Substances, as Soil Aggregators

Polyuronides and uronic groupings are present among humus constituents and these are widely distributed in plants, composts, and soils. The uronic acid content varies with the soil type [Bartholomew & Norman (10)] and is apparently related to its fertility. The polyuronides present include pectic acid, alginic acid, and various bacterial polysaccharides. Many mucilaginous polysaccharides contain uronic acid units, and it is known that complex polyuronides compose the plant gums found in soils containing decaying vegetable matter. Forsyth (11) has isolated from soil a polysaccharide fraction containing uronic acid units, and Fuller (23) has shown that uronides occur in humus.

Martin (12, 13) has found that the addition of *Bacillus subtilis* to soil produces aggregation of soil particles. This is due to the presence of organic substances resulting from the growth of the organism on a substrate such as sucrose. Levans are formed from sucrose by commonly occurring soil organisms (such as *B. subtilis*) which secrete an enzyme capable of levan synthesis (14). Martin demonstrated that bacterial polysaccharides such as the levans and dextrans are more effective than casein or lignin in improving soil aggregation [cf. McHenry & Russel (15)]. Geoghegan & Brian (16, 17) have more recently shown that bacterial polysaccharides aggregate soil [cf. Mc-

Calla (18)]. They conclude that bacterial levans and dextrans have marked aggregating affects on soil particles, the levans being formed by a wide variety of aerobic organisms and the dextrans being produced by various species of *Leuconostoc*. The aggregating action of the products derived from microorganisms exceeds that of the microbial cells themselves. As soil aggregators, dextrans, produced from sucrose by *Leuconsotoc*, are apparently superior to the levans, derived from sucrose by the action of *B. subtilis*. A polysaccharide formed by *Rhizobium*, containing 67 per cent glucose and 20 per cent uronic acid residues [Cooper *et al.* (19)] is a less efficient soil aggregator than either the levans or dextrans. Soil crumbs containing levans are very stable but are eventually broken down by bacterial action. The structure of the polysaccharide has a marked influence on the aggregation of soil particles, hydrogen bonding being considered an important mechanism whereby polysaccharides are bound to the soil particle [Geoghegan (20)]. Haworth *et al.* (21) have found that poor soils with a low total organic content contain only traces of polysaccharides, those with a higher total organic content have 0.5 to 1.5 g. polysaccharide per kilogram dry soil and possess a greater moisture retaining capacity. Polysaccharide fractions containing levans have been isolated from subsoils. Swaby (22), examining humus constituents, has shown that a variety of substances, including proteins and polyuronides, affect soil aggregation.

The aggregating effect of lignins, or lignin-like substances, in humus has been investigated by McCalla (24) and Alderfer *et al.* (25). Vallance (26) has recently reported that application of molasses to soils significantly increases the percentage of water stable aggregates. Kaila & Kivinen (27) have shown that straw composts in various stages of decomposition aggregate soils. Material containing more than 11 per cent hemicellulose and 18 per cent cellulose increases crumb formation. The results suggest that conditions favorable for microbial activity facilitate the improving action of the organic material on crumb formation but they also hasten the destruction of the formed aggregates.

The marked effect of a polyuronide, alginic acid, in increasing soil aeration by improving crumb stability and apparent water retention was first shown by Quastel & Webley (28) [see also Quastel (74)]. Using a manometric technique (29, 30) that measures directly the availability of oxygen to living cells in soil, they were able to demonstrate that the presence of alginates greatly affects the air-water relationship of soil. The amount of water that may be added to a soil before the availability of oxygen to the soil organisms falls through waterlogging and aggregate breakdown is much increased by the addition of sodium alginate. The addition of 1 per cent sodium alginate increases the aeration capacity of a poor soil to that found for a fertile garden soil. The effect of the alginate rapidly increases with increase of concentration to a maximum. Even a garden soil with a fairly high content of organic matter, showing good aeration at a high water content, is affected favorably by addition of alginate. A report on the improving effects of sodium alginate on

soil structure was submitted by the writer to the Agricultural Research Council (England) on January 10, 1942.

LABORATORY EVALUATION OF SOIL CONDITIONERS

The technique adopted by Quastel & Webley (28) for rapid evaluation of soil conditioners is based upon the employment of the Warburg manometric apparatus. A standard quantity of a crumbed, sieved, air-dried soil is placed in the flask of a Warburg respiratory vessel, and a known quantity of a suspension of microorganisms, e.g., baker's yeast (29) in a nutrient solution, is spread over the crumbs as evenly as possible. The rate of oxygen uptake at 37°C. by the microorganisms is measured. When oxygen is freely available to the organisms situated on the crumb surfaces, the rate of oxygen uptake is equal to that of the organisms suspended in nutrient solution in the absence of soil and examined under optimal respiratory conditions. As the volume of water in presence of the soil crumbs in the Warburg vessels is increased, oxygen availability becomes affected only when the water added is so large that the crumbs break down and the crumb pores and spaces become waterlogged. Under these conditions, the oxygen uptake of the microorganisms added to the soil drops sharply. The greater the crumb stability the greater is the quantity of water that may be added to the soil before the critical fall in the rate of oxygen absorption takes place. This technique provides a ready means of assessing the immediate effects of soil conditioners on crumb stability. The conditioners are added to a standard soil with sufficient water to form a thick mud which is allowed to dry at room temperature. This is crumbed and sieved before evaluation in the Warburg apparatus. The advantage of the technique lies in its yielding a direct measure of oxygen availability to the soil crumbs at various moisture contents. Moreover, since the technique is essentially a biological one depending on the respiratory activity of microorganisms, it provides data as to the possible toxicity of a proposed soil conditioner on these organisms. Clearly a conditioner that is highly toxic to soil organisms, poisoning their respiratory activity, is unlikely to find favor in agricultural practice.

The technique, however, involves special experience in the handling of Warburg manometric apparatus and is not as convenient for the screening of large numbers of substances as possible soil conditioners as the conventional methods of assessing soil aggregation. McCalla (24) has used a pipette method for investigating the effects of various substances on the aggregation of silt and clay particles. Geoghegan & Brian (17) have added solutions of various substances to a powdered soil, crumbs being formed by pressing the moist soil through a suitable sieve. After drying, the crumbs are agitated with water and wet-sieved. The proportion of stable aggregates is thus estimated. Hedrick & Mowry (31) have used both the manometric method and a modification of the wet-sieving technique of Yoder (32). They find that the wet-sieving technique, when suitably handled, gives results that provide excellent correlations with those obtained in the greenhouse and in the field.

The technique [see Hedrick (33)] consists in adding a soil conditioner to a standard quantity (100 g.) of pulverized Miami silt loam in presence of 30 per cent water, and pressing the resulting moist soil through a sieve to produce crumbs. These are dried and wet-sieved. The technique has been used for the comparison of thousands of substances tested as soil conditioners and gives reproducible results that compare well with those in the field.

Michaels & Lambe (34) have suggested a series of tests for the comparison of soil conditioners based on the behavior and properties of a soil suspension after treatment with the conditioners. Their procedures consist of flocculation and sedimentation tests, several aggregate-stability tests, water-permeability, and water-retention tests. They conclude that the possibility of developing a swift, simple, "screening" test for soil conditioners is remote, but that a series of suitable tests may aid greatly in the selection of suitable substances for field trial.

It should be pointed out that all the tests mentioned depend on the immediate, or very rapid, interaction of soil conditioners with soil particles. They give no information as to the stability of the conditioners (or of the complexes of conditioners with soil particles) under field conditions. Moreover, it is possible that a compound which, initially, reacts poorly with soil particles, may give rise under field conditions to substances with enhanced soil aggregating powers.

Effects of Cellulose Products on Soil Aeration

Using their manometric method, Quastel & Webley (28) showed that not only a polyuronide, such as alginate, is effective in improving soil structure, but that cellulose esters such as cellulose acetate, methyl cellulose and carboxymethyl cellulose will improve air-water relationships of a soil. These substances at concentrations of 1 per cent (per dry weight of soil) exercise effects similar to those due to about 0.5 per cent sodium alginate. The results are in harmony with those of Felber & Gardner (35, 36) who found that addition of methyl cellulose to soil secures considerable retention of moisture.

Addition of farmyard manure and horse dung greatly improves the air-water relationships and crumb stability of a soil. Quastel & Webley (28) point out that the effects of addition of these substances are twofold: (a) physical, due to the presence of fine straw which has a binding effect on the soil crumbs; the effect here is reversible, as washing the soil results in removal of the straw and the soil reverts largely to its original condition; (b) chemical, or physicochemical, due probably to the presence of polyuronides, or polysaccharides, affecting the soil particles; here the effect is irreversible as washing the soil causes no immediate diminution in crumb stability.

Addition of sewage sludges, composts, and refuses to soil improves the air-water relationships, usually in proportion to the amounts of organic matter present (28). Alkaline extracts of soils and peats, after subsequent neutralization, may also be effective.

ALGINATE AND SOIL PRODUCTIVITY

The finding that the addition of sodium alginate, at concentrations of over 0.1 per cent (per dry weight soil), had marked improving effects on soil structure led to an investigation of the effects of alginate on soil productivity. A series of experiments[2] by Quastel and Webley showed that, under glass-house conditions, the addition of alginate to a poor soil (a heavy clay depleted of organic matter) leads to an improvement in crop yield (tomatoes). This was confirmed by Owen (37), who investigated the relative effects of the salts of alginic acid (38), [see also Hedrick & Mowry (31)]. In the field, however, during a normal growing season, alginate application has apparently little or no beneficial effect on crop yield, and it became clear that the fairly rapid breakdown of the polyuronide in the soil militates against its use on a wide scale as a soil improver.

The conclusion reached by the reviewer in 1945 as a result of experiments with alginate, etc., was that it is unlikely that polyuronides, or polysaccharides, will be successful as soil improvers on a wide scale, though they may be of importance in the glasshouse where immediate improvements in soil structure may be very beneficial. For field work especially, substances must be sought that are less vulnerable to attack in the soil than the polyuronides and polysaccharides. These substances are metabolites of the cell, subject to breakdown by a variety of soil organisms. Their speed of decomposition in the soil, with consequent breakdown of the crumbs stabilized by such substances is determined, among other factors, by availability of nitrogenous compounds whose mobilization by the organisms attacking the polyuronides, etc., would also be deleterious to the plant. Moreover, the relatively large amounts of polyuronide salts required for structure improvement (5 to 10 tons per acre) would cause harmful effects to the soil due to the release of large quantities of cations present in the salts.

It became clear that the ideal soil conditioner should be a substance, having perhaps a similar mechanism to that of the polyuronides or polysaccharides in aggregating soil particles, but which would undergo a relatively slow rate of destruction in soil. It should, like the naturally occurring soil conditioners, be devoid of toxic effects on plant or animal. It should not interfere with the soil microbiological equilibria, involving, for example, the growth of nitrogen fixing organisms. Nor should it inhibit processes of soil nitrification; on the contrary, by improving soil aeration, it might accelerate them. It should not remove, at any rate irreversibly, the trace elements important for plant nutrition.

This ideal has been achieved to a great extent by the introduction of the synthetic polyelectrolytes by the Monsanto Chemical Company, with whose scientific staff the reviewer has had the privilege of being associated in the execution of this work. The first announcement of results with synthetic

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[2] In 1945 (unpublished).

polyelectrolytes was made at a meeting of the American Association for the Advancement of Science held in Philadelphia on December 29th, 1951.[3]

Effects of Synthetic Polyelectrolytes on Aggregation, Aeration, and Water Relationships of the Soil

Hedrick & Mowry (31), of the Scientific Staff of the Monsanto Chemical Co., using initially the manometric technique adopted by Quastel and Webley and later the wet-sieving technique (33), screened a large number of substances,[4] most of which were synthesized in the Monsanto laboratories. They found that only certain water-soluble polymeric electrolytes of high molecular weight are effective as soil aggregators, at the very low concentrations required by practical considerations. One of the most active substances was made by the hydrolysis of polyacrylonitrile. Other polymers of nearly related structure also showed great improving effects on soil aeration and soil aggregation. The two materials on which most work was reported were CRD-189, the sodium salt of hydrolyzed polyacrylonitrile; and CRD-186, another carboxylated polymer used as a partial calcium salt.[5] Both polymers are polyanions. Hedrick and Mowry showed that these substances, at concentrations of 0.1 per cent (per dry weight soil), give better aeration values, in a sandy loam, than any other materials tested (e.g., sodium alginate, sodium carboxymethyl cellulose, pectin, lignin) at 1 per cent. The aggregate stability is greatly increased, the effect being observed with many different soils. Very pronounced improvement in the workability of all soils treated with CRD-186 and CRD-189 takes place. They are crumbly and friable at high water contents. While the primary effect of the polyelectrolyte on the soil is on crumb stability, the percolation rate through treated soil often shows a hundred fold increase over untreated soil. The moisture equivalent is stated to be improved, and evidence from experiments on the wilting of plants indicates that all the increase in water held by the soil is available for plant growth. In addition to the more rapid infiltration and percolation of water, and increased moisture storage, a Miami silt loam treated with the synthetic conditioners showed decreased surface evaporation, an effect apparently due to the increased aggregate stability.

Allison (39) found from both laboratory and field studies that alkali soils from the Western United States, which are low in permeability, on treatment with CRD-186, give marked increases in permeability in proportion to the rate of treatment. High water stable aggregation in several saline and alkali soils is obtained after application of the conditioners at rates of 0.025 per cent and 0.1 per cent. Martin *et al.* (40) have found that application of CRD-186 and CRD-189 to heavy-textured Miami, Crosby, Brookston, and

[3] *Soil Science*, **72**, 419–92 (1952).

[4] Details of such substances may be found in U. S. Patents 2625529 and 2625471, issued Jan. 13, 1953.

[5] CRD-189 is also referred to as HPAN, and CRD-186 as VAMA.

Paulding soils in the field at rates varying from 0.02 to 0.2 percent in powder form, with subsequent mixing by disking and rototilling, increase soil aggregation and such related properties as porosity and permeability. The aggregates prove to be water stable, the structural improvement persisting through the second growing season. Bodman & Hagan (41), and Peters *et al.* (42) have found that the greatest absolute increase in aggregation of California soils on application of CRD-186 at 0.1 per cent are in the silt-loam, loam, and sandy-loam soils, the least pronounced effect being found with the finer textured soils which without treatment already possess more than 65 per cent by weight of water-stable aggregates. They point out that the addition of CRD-189 to Yolo soils ranging in texture from loamy-sand to clay, at the rate of 0.1 per cent or 0.2 per cent, produces no distinct change in moisture-equivalent or wilting point. The supply of water available to the plant may be increased, however, owing to the fact that the conditioners facilitate further infiltration of water and encourage deeper and more extensive plant root systems. Jamison (43) also notes that the polymer treatment has but little effect on wilting point and available water capacity, but points out that, under field conditions, improvements in soil structure influence soil-water relationships because increased aggregate stabilization provides increased water infiltration.

Vallance (26) [cf. Vallance & Leverington (44)] finds that treatment of poorly aggregated soil with Krilium (which is the trade mark of the Monsanto Chemical Co. for substances such as CRD-186 and CRD-189 sold as soil conditioners) at rates of 0.015 to 0.075 per cent increases water stable aggregates up to 53 to 70 per cent but there is little effect in well aggregated (55 per cent) soils. Ryan (45) points out, in connection with the culture of gladiolus, that heavy clay soils may be improved by the addition of the soil conditioner, preferably in solid form, but the soil must be in a good physical condition at the time of application of the conditioner.

It cannot be overemphasized that the soil conditioners such as polyuronides, or synthetic polyelectrolytes, stabilize soil crumbs and that, for their effective use, soils must be worked into a good structure before or at the time of application of the conditioner.

Kuipers & Boekel (46) find after application of Krilium to artificially aggregated samples of clay and loam soils, that in the clay soils there is an increase of the mean aggregate diameter, permeability, waterholding capacity and sticky point. Krilium produces a large increase in permeability of all soils, but the increases vary greatly with the different soils. Swanson (47) has also shown that Krilium administration improves aggregation, porosity and permeability, and prevents slaking; it stabilizes existing structure and facilitates drying. Improvements of soil structure of a cultivated loess by Krilium application have been noted by Hanotiaux & Manil (48) but there is apparently no improving effect on the surface layer of an acid and degraded forest soil. Increases of porosity and permeabilities of Ohio clays and silts after

application of Krilium have been reported by Martin & Volk (49) [see also Martin *et al.* (40); Martin (50)]. Allison (63) has found that application of CRD-186 effectively aggregates a Pachappa fine sandy loam and a Sebree subsoil, but is much less effective on a Sebree topsoil containing 3 per cent of 2-micron clay and otherwise chiefly silt and fine sand. It appears that the amount of clay in the soil is a dominating factor in determining the aggregating power of the polyelectrolyte. As determined by permeability measurements, the polyelectrolyte treatment, at both 0.025 and 0.100 per cent levels, seems to overcome the dispersive effect of a high content of exhangeable sodium. In a comparison of aggregating effects of CRD-186 and CRD-189 on nine western soils, varying widely in a number of chemical and physical characteristics, it was found that regardless of pH, salinity, exchangeable-sodium percentage, or clay content of the various soils tested, there seems to be no significant difference between the abilities of the two polyelectrolyte conditioners to produce water-stable aggregates.

Administration of CRD-186 to alkaline and saline soils facilitates removal of salt and exchangeable sodium after soil irrigation, a property of considerable importance in soil reclamation.

Kinds of Soil Conditioners and Formulations

Gardner (51) presents a useful list of the trade names, physical form and percentage of active conditioning material in various substances sold as conditioners. Most of the legitimate soil conditioning materials have as their basis some form of hydrolyzed polyacrylonitrile (HPAN, as an abbreviation) or a modified vinylacetate-maleic acid compound (VAMA). The Krilium formulations contain one or other of these products. In addition to these materials, there are other chemical, mineral, organic, and biological products claimed to have properties similar to those of the synthetic polyelectrolytes. Gardner points out that many of the claims made for large numbers of soil conditioners now on the market are grossly exaggerated and misleading, and Nason (52, 53) has emphasized the fact that some of the formulations at present on the market are either deficient in active ingredients or contain substances that may lose their activity in a relatively short period of time.

Various silicates and silicones, resistant to attack by microorganisms, have been investiaged as soil aggregators (54, 55, 56, 57). These have facilitated soil aggregations but they give rise to water-proofing effects (and high alkalinity in the case of sodium silicate) or are impracticable as with the volatile silicones. Martin (50) considers, however, that they are worthy of more extensive field testing, in view of certain beneficial effects of sodium silicate on grass yield on a calcareous Houston clay [Laws (55)]. The acid salts of iron and aluminum have been used as soil conditioners apparently with some success in Italy (62). A flocculating action of these trivalent ions, together with acidity, cause increased friability and permeability in dense clays.

Application of Soil Conditioners

Thorough mixing of the synthetic soil conditioners with a soil is essential for the stabilisation of structure. Disking or rototilling for incorporation is satisfactory (50), and the soils should contain sufficient moisture for good workability. If soils are too wet, mixing is difficult owing to gumming and, if too dry, the soil should be remixed after irrigation. Conditioners prepared in liquid form are very effective in prepared seedbeds for prevention of crusting or erosion. They are useful also for surface row application in commercial scale agriculture (53) and may be applied with conventional farm spray equipment.

The texture of a soil influences the efficiency of a conditioner as a soil aggregator. Greater aggregation is obtained in a fine textured soil than in one of coarse texture. VAMA conditioner is more effective on illite and kaolinite than on calcium bentonite and it will also affect fine quartz particles (50). Particle size, organic matter content, salt content, and pH, all influence the aggregating properties of soil conditioners.

In general, soils of high clay content, that compact and crust markedly, respond best to treatment with synthetic polyelectrolytes. But various degrees of structural response have been obtained on soils of every textural class, regardless of the initial state of soil structure (58).

Stability of Synthetic Soil Conditioners in Soils

Hedrick & Mowry (31) pointed out that the synthetic polyelectrolytes are highly resistant to attack by microorganisms. Using the soil perfusion apparatus of Lees & Quastel (59), as modified by Audus (60), they were able to show persistence of conditioner stabilized aggregates after treatment with 0.1 per cent CRD-186 for 32 months at 76°F., a far longer period than was obtained after treatment with 1 per cent sodium alginate and with a dried compost. They showed, too, that little or no loss of conditioner took place after thorough leaching of the treated crumbs for 6 weeks. Martin (50) reports that preliminary tests with C^{14}-labelled polymers indicate that the polymers combine with the soil particles very quickly in solution and do not move appreciably afterwards. When dry-mixed and then leached, movement of the polymer occurs probably during the solution process. Radioactive carbon was not detected in the leachate from a 16 in. soil column through which passed 39 in. of water. Incubation of a Brookston silty clay loam with C^{14}-labelled HPAN at 27°C. with optimum moisture resulted in the production after 39 days of $C^{14}O_2$ equivalent to 0.97 per cent of the conditioner, 13 per cent of this being produced in the first 24 hr. of incubation. Fuller & Gairaud (61), in a study of the effects of soil conditioners on CO_2 evolution from soils, find that a small proportion of VAMA, and a lesser proportion of HPAN, is available to attack by soil microflora, the remaining material being so slowly available for decomposition that it has no measurable influence on the CO_2 evolved during a period of five months. Field tests also indicate the marked

persistence of the aggregations caused by the synthetic polyelectrolytes, but plowing and cultivation result ultimately in crumb breakdown (50). In tests where VAMA at the rate of 0.05 per cent was added annually for 3 years to Brookston clay loam in a plow layer application, appreciable breakdown of aggregates occurred each year over the levels attained at the time of application. Aggregation at the end of the third season, however, was greater than at the commencement, i.e., 46 per cent in 1950, 54 per cent in 1951, and 65 per cent in 1952, the controls being 30 per cent, 26 per cent, and 32 per cent for the same dates respectively [Martin (50)]. In tests using higher quantities of VAMA with correspondingly higher percentage of aggregations there was a substantial drop in the aggregation after the second season, the treated soils at this time, however, being considerably better aggregated than the controls.

CROP RESPONSE TO SYNTHETIC SOIL CONDITIONERS

The fact that soil treated with a conditioner retains its porous friable structure under conditions of excess moisture and heat, with such benefits as increased aeration, drainage, and decreased surface soil dispersion and crusting, is reflected in improved crop responses. The improvements in soil structure lead usually to increased rates of germination, emergence and root formation, more rapid early growth, and better crop yields and quality. Crop increases, however, may not always take place following conditioner treatment, as soil structure is not always the limiting factor. Deficiencies of water or nutrients may, for example, limit the crop response. It is, of course, important to note that the synthetic polyelectrolytes have themselves no nutrient value to crops. Their sole importance lies in their improvement, or stabilization, of soil structure. Hedrick & Mowry (31) noted the improving effect, on the growth of radishes, due to the application of CRD-189 to a Miami silt loam. Moreover, the synthetic polymer (in absence of added nutrients) showed no inhibitive effects on the plant growth, as would have been expected had it been broken down by soil microflora with mobilization of available nitrogen.

According to Allison (63) the application of CRD-186 and CRD-189 have favorable effects on crop yields in several alkali and saline soils from the Western U.S.A. Sweet corn was grown on treated and untreated soils; whereas the untreated soils gave poor stands of corn owing to heavy crust formation, full stands were obtained on the treated soils. The quality of corn was excellent and yields were good despite the prevailing high temperatures during the tests. Martin *et al.* (40) found that corn, oats, and carrots are most responsive to conditioner treatment, no toxicities being apparent. Treatments of disk-mixed and rototilled plots (Miami silt loam) with CRD-186 increase yields of corn substantially over the control, the yield difference between rates of application of 0.02 per cent and 0.1 per cent of the conditioner not being significant. Top dress application before cultivation is ineffective in increasing corn yields. Little correlation exists between percentage aggrega-

tion and crop yield. It is to be noted that conditioner treatment stabilizes large aggregates so that water infiltration, aeration and permeability are all improved and these factors are not necessarily reflected in the assessment of percentage aggregation. Using turnips on a Brookston clay loam, Martin *et al.* (40) found that early growth of the crop is substantially better in treated soils than in the control soils at the time of harvest. The root weights, however, are essentially the same. Better foliage development is reflected in a significantly higher weight of green leaves even at harvest time. As with all conditioner treated soils, it was observed that cultivation, weeding, harvesting, and digging operations are considerably easier with the treated soils, which are crumbly in contrast to the lumpy and cloddy character of the untreated soils. With potatoes, although the average yield seems to be increased by the conditioner treatment, differences are not statistically significant. With carrots, statistically important yield increases are observed. On Paulding clay soils in Ohio, conditioner treatment results in a doubling of height of sweet corn and substantial weight increases in unthinned red beets. Oats planted as a nurse crop for alfalfa on Crosby silt loam yield 40 per cent more on treated soils.

Germination and emergence improvement, as a result of conditioner treatment, have been noted with cotton, soya beans, sugar beets, peas, lettuce, tomatoes, snap beans, alfalfa, and lettuce (53). Successful results have been obtained by conditioner treatment in the glasshouse and nursery field, in the growing of dahlias, poinsettias, roses, cyclamen, hydrangea, calcoelaria, lilies, solanum, and cymbidium (53). Depth treatment with soil conditioner of tobacco seed beds has resulted in the growth of a larger number of plants satisfactory for transplanting, a principle that is being applied to other plants. Wester (64) has obtained significant early yield increases of broccoli, lettuce, cabbage, and tomatoes as a result of conditioner treatment in seedbed flats and in individual hills. Early tomato yields were increased by 79 per cent by HPAN treatment and 154 per cent by VAMA incorporation. Substantial improvements as a result of treatment were also noted in midseason yield totals but the late season totals were not affected by the conditioner treatment. Engibous & Deming (65), reporting on over 40 randomized, replicated series of tests involving soil conditioner treatments in tobacco plant beds, conclude that conditioners based on VAMA are superior to HPAN in aggregate stabilizing effects, soil compacting and crusting being largely eliminated. There is an increased plant stand in the seed bed and increased seedling vigor as shown by the improved results over the controls following transplanting. Results similar to those obtained with tobacco in vegetable crop seed beds are noted with tomatoes, lettuce, cauliflower, cabbage, and peppers [see also Szasz *et al.* (66)] Cutflower growers are now taking advantage of this development in conditioner application to produce more vigorous transplants (e.g., with chrysanthemums and carnations). Engibous & Deming (65) point out that under conditions of soil crusting, emergence of crops is aided by surface band application of soil conditioners at the time of planting;

this may result in significant yield increases. Fuller & Cords (67) have reported that both stand and yield of alfalfa and sour clover are increased in field crops by application of HPAN. Fuller *et al.* (68) conclude that the stand of corn and sudan grass on calcareous soils of Southern Arizona is significantly improved by conditioner treatment. The yield of sweet corn roasting ears, lint cotton, grain sorghum, and sudan grass is significantly higher in the soils treated with the polyelectrolytes, but there is no significant difference in the yield of barley from the control. In general, the application of VAMA in concentrations of four tons per acre has no greater influence on plant growth or population than an application of one ton per acre. Improvements in crops have been noted with applications of VAMA and HPAN at concentrations as low as 200 pounds per acre. Fuller *et al.* (68) note that the nitrogen and phosphorous content of barley grain, cotton leaves and petioles, and hegari leaves are higher from plants grown in soils treated with conditioners than from those grown in the control soils. The nitrogen and phosphorus content of either barley tops or hay of sudan grass are not changed.

Swanson (47) has pointed out that application of the polyelectrolyte at the rate of 4000 lb./acre to a loamy sand in Connecticut increased lettuce yield by 325 per cent. Martin & Volk (49) have also noted the improvement in growth and yields of several vegetable crops as a result of VAMA application, together with an earlier maturation of tomatoes and increased efficiency of applied nitrogen fertilizers.

Effects of Synthetic Soil Conditioners on Nutrient Availability

Bould & Tolhurst (69) find that application of the sodium salt of hydrolyzed polyacrylonitrile has no effect on exchangeable potassium or exchangeable magnesium of a soil when used over the range 0.05 to 2 per cent on a dry weight basis. The polymer has no effect either on available soil potassium but increases available soil phosphate, the latter effect increasing with increase of rate of application. The polymer also has no significant over-all effect on the uptake of potassium and phosphate from soil by rye seedlings grown in plots in the presence and absence of added K and P fertilizers. Spectroscopic evidence (31) derived from analysis of kidney beans, wheat, and radish has already shown that nutrients and trace elements are not rendered unavailable by conditioner treatment. Plant tissue analyses show that so far as nitrogen, phosphorus, and potassium are concerned, soil conditioners do not influence nutrient uptake (50). Sherwood & Engibous (58) report, however, that availability of mineral elements may be increased by application of soil conditioners. In one case, corn growing on treated soils absorbed more phosphorus than on untreated soil, but rye grass did not. This was reversed with respect to the absorption of nitrogen. Neither species showed increased potassium absorption.

Compatibility of Soil Conditioners with Fertilizers

Martin (50) points out that if fertilizer salts such as monopotassium phosphate, potassium chloride, or ammonium nitrate are first mixed with a poly-

electrolyte conditioner, some reduction in aggregating value may take place. He concludes that phosphates and certain organic anions, like citrate, seem to be compatible at normal levels of application with both VAMA and HPAN and that admixture of these substances diminishes the effects of nitrates, chlorides, and sulphates.

Soil Erosion

Equal in importance to the experiments showing the effects of soil conditioners on the improvements of crop yields in various soils are those demonstrating the effects of conditioners in the control of soil erosion. Weeks & Colter (70) have shown that surface soil can be satisfactorily stabilized to the erosive action of rainfall by treating the surface with the soil conditioner. The permeable film produced by the conditioners not only stabilizes the soil but prevents run-off. The effect is much the same as that due to the application of a high rate of straw mulch. Addition of half to one pound of the polyelectrolyte to 100 square feet may provide satisfactory protection to the soil. One set of results on the effects of artificial rainfall on experimental plots showed that erosion equal to a loss of 50 tons of soil per acre was reduced to a loss of 3 tons per acre by application of the soil conditioner [see also Goodman (71)].

Engibous & Deming (65) point out that spray truck application of polyelectrolyte, seed and fertilizer offer a simple means of roadbank stabilization. Very early spring plantings are made possible in which straw mulch covers to provide shading are not needed. They conclude that there is a rather general superiority of maleic polymers for soil stabilization. Effective erosion control and grass establishment are obtained with low rates of polymer application (e.g., 100 lb. per acre). Martin (50) reports a substantial diminution of soil erosion and run-off by application of HPAN at the rate 0.05 per cent disked into the top 4 or 5 in. of soil. He points out that because of a tendency of conditioner treated and well aggregated soils to dry out on the soil surface, the conditioners cannot completely take the place of organic mulch even though serious erosion may be prevented.

Toxicity of Soil Conditioners

The synthetic polyelectrolytes seem not to be toxic to earthworms, which appear in even greater numbers in the more friable well aerated soils produced by the conditioner. Rats and chicks have been fed with relatively large doses of these conditioners with no apparent ill effect. Nitrification studies indicate no detrimental effect upon organisms responsible for this process in soil (31). Sherwood & Engibous (58) also claim nontoxicity of the electrolytes when fed to rats in doses of 3 g. per kilo body weight.

Mechanism of Action of Synthetic Polyelectrolytes

Ruehrwein & Ward (72) have studied (a) x-ray diffraction by montmorillonite clay treated with polyelectrolytes, (b) measurements of adsorption of polymers by kaolinite clay, and (c) flocculation of clay suspensions by pol-

ymers. The polymers employed were sodium polymethacrylate, a representative polyanion, and the acetic acid salt of polyβ-dimethylaminoethylmethacrylate as a representative polycation, both being made by conventional vinyl polymerization techniques. These workers conclude that polycations, but not polyanions, are adsorbed in the interplanar spacing of the expanding lattice clay montmorillonite, probably by cation exchange. Sodium polymethacrylate is adsorbed on kaolinite up to a level of 2 me. per 100 g., the adsorption being about three-quarters complete in one day. The degree of adsorption depends on the concentration of extraneous electrolyte. It acts as a clay aggregate stabilizing agent binding the particles together. The polymer molecules are sufficiently long to bridge the gap between the clay particles and they are capable of being strongly absorbed on the clay to form anchor points for the bridge. The adsorption process may be one of ion exchange or replacement. The clay particles are loosely bound by only a small amount of polymer, the aggregates being stable and not dispersed by water.

Doubtless, the further study of polyelectrolyte interaction with soil constituents will involve investigation of the properties, particularly the surface activities, of polyelectrolytes under a variety of conditions. Katchalsky & Miller (73) have investigated the dependence of the surface activities of polymethacrylic acid on the molecular weight of the polymer and on the pH and ionic strength of the medium and have shown that the surface activity decreases with the inverse square root of the molecular weight. Viscometric measurements in acid solution indicate that the polymer is hypercoiled, this being due to strong intramolecular hydrogen bonding of the carboxyl groups.

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THE PHOTOSYNTHETIC FUNCTION OF PIGMENTS OTHER THAN CHLOROPHYLL[1]

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Ever since men noticed yellowing autumnal leaves, or gathered brown, red, or blue algae, they must have speculated as to the meaning of these colors. Scientific interest became the more acute as physiologists realized the universal distribution of chlorophyll in photosynthetic systems, and its undoubted importance as the primary light absorber. But the other pigments are also widespread: one (β-carotene) is almost always present, and there are usually several others found in every photosynthetic system. Their distribution is remarkably parallel to phylogenetic lines.

What are these other pigments doing? Are they accidental and indifferent, merely happening to absorb light (like hemoglobin) without this absorption having much physiological function? Are they helpful or wasteful filters, by their absorption protecting other systems from injurious or useful radiation? Are they, perhaps, themselves injurious, inducing photodynamic or photooxidative effects? Do they collaborate with chlorophyll, optically or chemically, thereby qualifying as truly "accessory"? Or are they completely independent, supplementary light absorbers, with their own parallel complement of enzyme systems, able to carry out photosynthesis without the intervention of chlorophyll? Indeed, do they sometimes take over the major absorbing function, subordinating chlorophyll itself to an accessory or indifferent position?

This chapter attempts to summarize current information on the role of the so-called "accessory pigments" in photosynthesis. There has been brief mention of these in various recent reviews (1 to 5), and Rabinowitch has given his usual excellent discussion of the problem [(6), chaps. 15, 17; (7), chaps. 21–24, 30]. Several comprehensive descriptions of the pigments, without much emphasis on their function, have appeared [Boresch (8); Cook (9); Strain, (10, 11, 12)]. The present author has assembled some of the background, as well as new material, especially on the algae, in a forthcoming chapter [Blinks (13)]. No attempt is made here to include photosynthetic bacteria, which will be covered in Volume 8 of the *Annual Review of Microbiology*.

THE PIGMENTS

Carotenoids.—Some of these are probably in the inactive category, but others are definitely "accessory." Their chemical nature is extensively discussed by Strain (14), Karrer & Jucker (15), and Zechmeister (16). Carote-

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[1] The survey of the literature pertaining to this review was concluded in September, 1953.

noids are often found in plastids, but sometimes in separate oil globules, which topographically excludes them from participation in photosynthesis (see below). When they are in the plastid, only experiment can decide whether they are active or inactive, and the answer is not consistent.

Phycobilins.—As their name implies, these accessory pigments are found only in the algae. They were so named by Lemberg (17 to 20) who showed their chemical affinity to the bile pigments. A résumé of their structure is given by Lemberg & Legge (21). Though water soluble, the phycobilins are found in the plastids of red algae, and probably in the "grana" of blue-green algae.

Connection to proteins.—Both chlorophyll and carotenoids are now generally assumed to be conjugated with proteins in the plastid, and some studies have been made of such systems *in vitro*. But the connection is relatively feeble, and is easily split by gentle warming, and by solvents such as methyl alcohol. The phycobilins, on the contrary, are remarkably stable chromoproteins; water soluble, crystallizable, of definite molecular weight (270,000), having characteristic isoelectric point and mobility, diffusion and adsorbability [Kylin (22); Svedberg *et al.*, (23, 24, 25); Tiselius (26); Swingle & Tiselius (27)]. They were, indeed, among the earliest proteins to be studied physico-chemically, because of their stability and color. Their amino acid composition has also been investigated; they resemble general cytoplasmic proteins in this regard [Kitasato (28); Wassink & Ragetli (29).] A possible phosphate attachment has also been recently suggested [Blinks (13)].

TABLE I

MAJOR ABSORPTION REGIONS OF THE PRINCIPAL PLANT PIGMENTS
(mμ)

	VIOLET,	BLUE,	BLUE-GREEN,	GREEN,	YELLOW,	ORANGE,	RED
Chlorophyll-*a*	435						670
β-carotene		425, 450, 475					
fucoxanthin		452, 490					
R-phycoerythrin			495	540	565		
B- "				550	565		
C- "				550			
R-phycocyanin				(550)		615	
C- "						615	
P- "							650

The absorption maxima of the first three are dependent on the solvent. Those given are rather generalized, and do not exactly represent the absorption in the plant. Chlorophylls-*b,c,d*, and many carotenoids omitted.

Absorption.—As shown in Table I, the carotenoids overlap chlorophyll's absorption in the violet and blue end of the spectrum, though with broader zones. This, together with the slightly different absorption maxima of the

individual carotenoids present, makes decision often difficult as to their role, and especially as to which particular carotenoid is implicated. The situation is easier to analyze in the case of the important carotenoid, fucoxanthin, which absorbs appreciably farther into the green, in living diatoms and kelps,—though not in dead cells or extracts. This change is ascribed to the breaking of the chromoprotein linkage (see below).

Phycoerythrins are lavender red, and almost completely complementary in color to chlorophyll, closing in the gap in the middle of the spectrum. R-phycoerythrin (from the Rhodophyta, the higher red algae), has three absorption maxima, at 495, 540, and 565 mμ, varying somewhat in height depending on the algal source (25). C-phycoerythrin (from the Cyanophyta, the blue-green algae) has but one absorption peak, close to 550 mμ; while a newly isolated pigment, tentatively designated B-phycoerythrin (from a member of the primitive red algae, Bangiales) rather resembles it but has two peaks, a major one at 550 mμ and a small one at 565 mμ [Blinks (13)]. All of these transmit well in the blue end of the spectrum, and almost completely in the red end. There is little overlap with chlorophyll-a or carotenoids.

The remaining spectral region where none of the preceding pigments absorb very strongly is the orange red (600 to 640 mμ). The gap, however, is filled by C-phycocyanin, with absorption maximum at 615 mμ. This is the characteristic pigment of the blue-green algae (Cyanophyta); it is also found in *Porphyra naiadum* (13). Another type, R-phycocyanin, prevails in most of the Rhodophyta (higher red algae); in addition to its principal maximum at 615 mμ, it has a smaller absorption peak at 550 mμ.

There is now good evidence of a new phycobilin, P-phycocyanin, isolated from a number of blue-green and red algae by Haxo, O'h Eocha, and Strout [quoted in Blinks (13)]. This has an absorption maximum at 650μ.

Inactive pigments.—Some of the common colored substances of plants can readily be dismissed from photosynthetic connection (save as end products). These include anthocyanins, flavones, and tannins. These are usually in vacuoles, either in solution or precipitated, and are at such a distance from the plastids, either in separate cells or at least isolated by the tonoplast, as to have little chance of passing absorbed energy into the photosynthetic system. If they have any function it is trivial, merely as filters cutting off light from the other pigments [cf. Strain (30)].

Pigments of unknown function.—Two rare water-soluble pigments have been found in algae: fucosan [Kylin (31)] and floridorubin [Feldmann (32)]. Their function is completely unknown. Fucosan, being in special cells (31), is probably inactive photosynthetically.

Riboflavin, hematins (33), and the many fluorescent substances recently tabulated by Goodwin (34), are probably not sufficiently concentrated to be regarded as pigments, absorbing light appreciably. Yet many are doubtless important in photosynthesis, and may prove to have very interesting effects at their characteristic absorption maxima. Some of the unexplained peculiar-

ities of action spectra or quantum efficiency may find their origin in these "minor" absorptions.

Algae

For various reasons, algae have become the favored, almost the "standard" plants for photosynthetic study. Optically they facilitate the determination of absorption spectra, because thin thalli (often one cell thick) or dilute suspensions of cells can be used, with little reflection, scattering, or repeated absorption paths. Cellular environments can be readily controlled, and gas exchange facilitated by shaking, or obviated by determinations in solution. They also have the greatest range of pigments.

Green algae.—The simplest, and to this day perhaps the most sensitive method of determining photosynthetic action spectra was developed by Engelmann (35, 36, 37). He introduced a suspension of bacteria around a *Spirogyra* filament; these bacteria congregated where the oxygen content of the water was highest (detecting 10^{-12} milligram of oxygen!). When the *Spirogyra* was illuminated by a micro-spectrum, the bacteria soon moved to two main regions, the blue and red parts of the spectrum. Their number was a measure of the oxygen production; when plotted it gave the first *action spectrum*. This corresponded on the whole with the absorption spectrum, with maxima both in red and blue, but the latter was distinctly lower (about 65 per cent of the former). Though not specifically discussed by Engelmann, this probably indicates a degree of inactive absorption by carotenoids.

This was the conclusion reached sixty years later by Emerson & Lewis (38). In their careful study of the quantum efficiency of *Chlorella*, they found rather constant values (0.09–0.1 molecules O_2 per absorbed quantum) in the red end of the spectrum, but consistently lower yields (0.08) in the blue, and still lower ones (down to 0.065) in the blue-green (480 to 490 mμ). The fall of efficiency was ascribed by the authors to inactive carotenoid absorption, and this is doubtless correct. But such absorption even in the blue-green region, can hardly be entirely inefficient, for, as Rabinowitch points out, carotenoids are here absorbing 60 to 70 per cent of the light, yet efficiency is down only about 25 per cent from the chlorophyll maxima in the red. The situation is still better at 420 mμ, where carotenoids absorb about 50 per cent of the light; yet efficiency is only about 10 per cent less than in the red.

Nevertheless, the depression around 480 mμ is extremely interesting, and appears again in the action spectra taken by a totally different method (polarographically or amperometrically) by Haxo & Blinks (39). Using thin thalli of the sea lettuce *Ulva* (two cells thick), detailed action spectra were obtained at some thirty points throughout the visible spectrum (Fig. 1) and compared with the absorption of the same thallus. Deviations occurred at three regions: above 700 mμ, where, as Emerson & Lewis had found (38), chlorophyll absorption was almost completely inefficient; in the middle of the spectrum around 540 mμ, where we now know the absorption determina-

tions were somewhat too high (Yocum (40)] and in the blue-green region just discussed (460 to 500 mμ).

Otherwise photosynthesis paralleled absorption almost perfectly, even at the blue chlorophyll peak (435 mμ), where almost half the absorption (in extracts) is due to carotenoids. At this point it seems completely equivalent whether the energy is absorbed by chlorophyll or carotenoids; it is used equally well. Yocum (40) pointed out however, that not all samples of *Ulva*
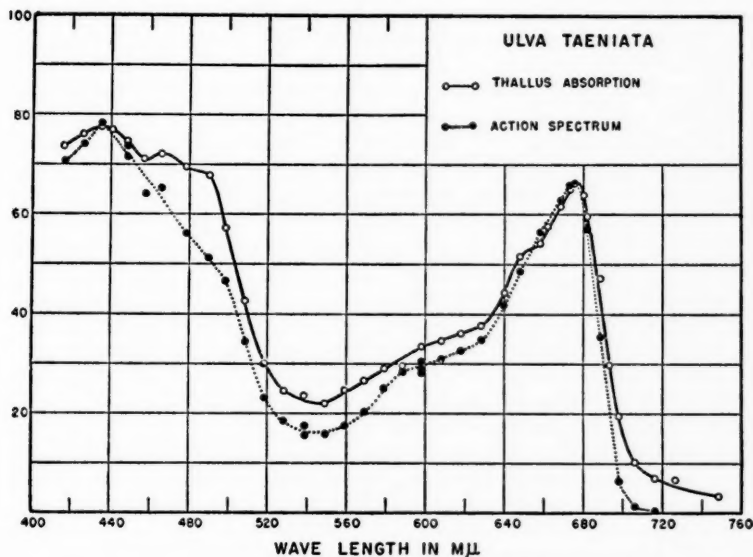
FIG. 1. Absorption and action spectra for the green alga *Ulva*. The solid line is per cent absorption. The dotted line is relative photosynthetic rate (for equal incident quanta). This is made to correspond with absorption at 675 mμ. Good correspondence between the curves indicates efficient participation of all the pigments except in the region 460–500 mμ, and beyond 700 mμ [after Haxo & Blinks (39)].

showed this high photosynthetic rate in the blue; apparently carotenoids are sometimes considerably less efficient. The reasons for this should be explored further. It is partly seasonal, and may be connected with reproductive cycles. Haxo & Clendenning (41) have shown that the fruiting margins of *Ulva* (before release of gametes) have a higher carotenoid content (largely γ-carotene), but lower photosynthetic activity than the adjacent vegetative cells. The female gametes themselves had about the same photosynthetic rate as the males, but since their chlorophyll was twice as concentrated, their

photosynthetic rate per unit of chlorophyll was only about half as high. While no action spectra were taken, this material might yield much information as to the relative roles of chlorophyll and carotenoids. These varied in the ratios 8.3, 2.7, and 1.1 in the different cells. It would be important to determine, however, that the accumulation of carotenoids was actually in plastids, rather than in eye spots or oil globules (see below).

Another approach which might yield interesting results would be to determine the action spectra of mutant strains, such as the *Chlorella* series studied by Granick (42). Among these might be algae with more active or completely inactive, carotenoids—not to mention degrees of chlorophyll activity.

There are also certain highly specialized green algae which use their total carotenoids far less efficiently than *Spirogyra*, *Chlorella*, or *Ulva*. These are the "orange" algae, such as *Haematococcus*, *Trentepohlia*, and *Dunaliella*. They are really green algae heavily charged with oil globules in which β-carotene is dissolved. It is not clear why all these should inhabit difficult ecological niches (snow banks, spray-swept trees, and saturated brines); the high carotene content may be a response to desiccating conditions or high intensities of light. In any case, these algae scarcely use blue light at all; we have found that their photosynthesis is completely restricted to the red end of the spectrum (13). This is probably a simple shading effect, blue light being absorbed by the abundant carotene in separate structures before it can reach the chlorophyll. "Green" *Dunaliella*, grown on less concentrated brine, uses blue light more effectively [cf. Fox & Sargent (43) for the relative chlorophyll and carotenoid content of such cultures].

There is, however, another possibility: photooxidation. Not only is there no oxygen evolution, but sometimes there is an enhanced respiration when these orange algae are exposed to blue light. This has been observed after such exposures in *Chlorella* [Emerson & Lewis (38)]; conceivably the effect might be responsible for the apparently lower photosynthetic rate in that region of the spectrum. An answer should be easily obtained with the mass-spectrograph.

Brown algae and diatoms.—In contrast to the variable, and sometimes completely inactive, role of carotenoids in the green algae, the brown algae and diatoms supply our most clear-cut example of effective collaboration of carotenoids with chlorophyll. The story again begins with Engelmann (37) who exposed diatoms to a micro-spectrum and found bacteria congregated at both its ends. But now blue light was 80 per cent as effective as red, and furthermore, the effectiveness of blue-green light was greatly enhanced, with a broad photosynthetic maximum from 520 to 560 mμ, 92 to 95 per cent as high as the red value. Quite clearly the total efficiency in the shorter wave lengths was very good, and the usable range has been markedly extended toward the middle of the spectrum.

Engelmann's striking results have been amply confirmed by a large number of workers, both with diatoms and with larger brown algae. Montfort (45 to 48), Schmidt (49), and Levring (50) all used the larger brown algae, and similar procedures: (algae in closed bottles of sea water, illuminated with filtered sunlight or artificial light, sometimes submerged in the ocean). Oxygen determinations by the Winkler technique, at the end of the illumination period, gave an indication of photosynthetic rates. The technique has been criticized [Emerson & Green (51)] because of inadequate CO_2 supply; light absorption was also uncertain, and usually not measured. However, the results have some comparative value between spectral regions, and between species. Compared to green algae, many of the brown algae showed enhanced photosynthesis in the blue end of the spectrum; indeed, the rate was often higher than in the red end. This is probably due to the very broad spectral zones passed by the filters, and a broader zone of absorption, extending into the blue-green region, rather than to enhanced activity at the blue chlorophyll maximum itself. The conclusion was drawn that carotenoids assisted photosynthesis, and, because of its high concentration, fucoxanthin was indicated as the responsible carotenoid. Actually, most of these results were not detailed enough to support this latter inference, correct as it probably is. Only the curves of Levring had sufficient detail to support what Engelmann could clearly see: that activity was extended distinctly into the green, where fucoxanthin does indeed absorb. Even Levring's results were obtained by rather broad filters (for which he took the "center of gravity" of transmission) rather than by reasonably monochromatic light.

Fortunately, the bright mercury line at 496 mμ, and the green one at 546 mμ are so located that fucoxanthin (and other carotenoids) absorb respectively 93 per cent and 48 per cent of the light in extracts of brown algae (chlorophyll absorbing the rest). Dutton & Manning (52) found that when diatoms were illuminated from these sources at wave lengths largely absorbed by fucoxanthin, photosynthesis was almost as great as when they received blue light (435 mμ) or red (centered at 665 mμ) of equally absorbed energy. They concluded that the carotenoids of diatoms (especially fucoxanthin) absorb energy for photosynthesis as effectively as chlorophyll itself. This conclusion was confirmed in diatoms by Wassink & Kersten (53) and by Sagromsky (54). Tanada (55) in a very detailed study, showed that the quantum efficiency of diatoms was nearly equal (about 0.11) throughout the visible spectrum (except in the already familiar "depressed zone" around 480 mμ). This could not be the case unless the energy absorbed by (all) the carotenoids was used as effectively as that absorbed by chlorophyll alone. Fucoxanthin probably aids chiefly by increasing the absorption toward the center of the spectrum; in living brown algae this is considerably increased in the region 500 to 560 mμ. The displacement is probably due to a fucoxanthin-protein [Menke (56), Wassink & Kersten (53)]. This complex is very
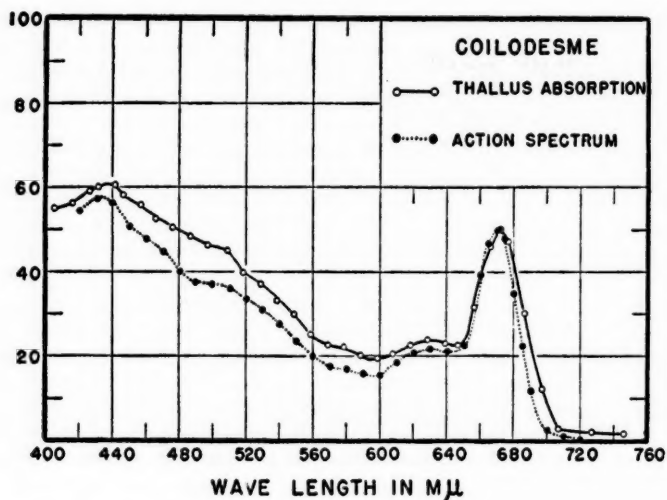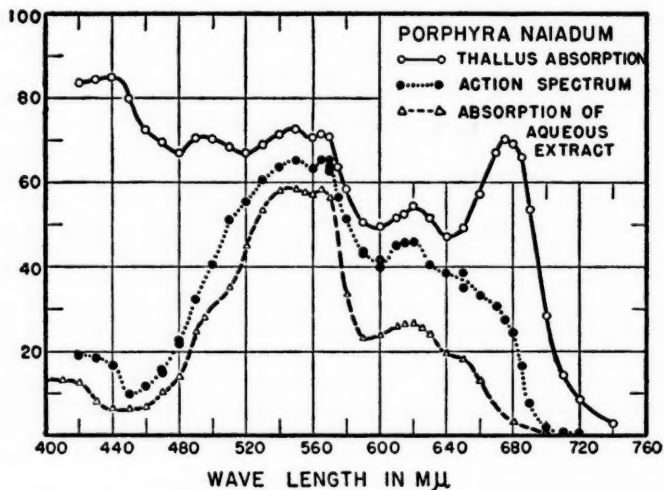
FIG. 2. Action and absorption spectra like that of Figure 1, but for a brown alga greatly in the red algae, where phycoerythrin and phycocyanin are the more active show absorption spectra of the water-soluble pigments (phycobilins). Note increased
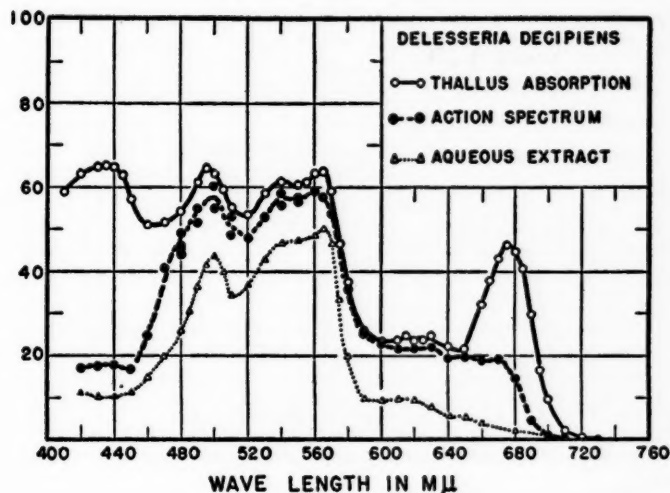
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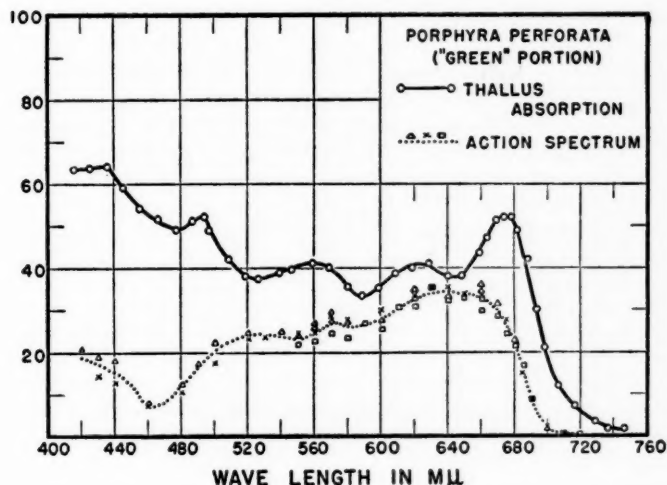
d

and three red algae. The two curves agree well in the brown alga (*a*), but deviate
pigments. Chlorophyll and carotenoid activity is markedly depressed. (*b*) and (*c*)
absorption in (*c*) and (*d*) at 620 mμ due to phycocyanin [after **Haxo** & Blinks (39)].

labile and on warming, drying, acidification, or exposure to alcohol, diatoms and kelps immediately turn green. This is due to a decreased absorption in the region 500 to 560 mμ, as indicated by the curve for normal and heated *Laminaria* [Haxo & Blinks, (39)]. Green and red algae do not show this effect on warming.

The action spectrum of a thalloid brown alga (*Coilodesme*) shows especially well how fucoxanthin absorption in the blue-green alters the action spectrum (Fig. 2a). This resembles that of *Ulva* except for an enhanced activity between 500 and 560 mμ; action parallels absorption almost completely except for the region 480 to 490 mμ, where again action is appreciably lower. If this is due to some other carotenoid, then not even the brown algae have completely activated these pigments. But they have come very near to this, and it is perhaps not surprising that diatoms, in numbers, and kelps, in size, are among the most successful of algae, especially in the ocean. They absorb fairly completely through the spectrum and utilize most wave lengths of the absorbed light. Where individual cells still transmit, in the yellow, orange and green regions, increased thickness often enhances absorption, rendering some of the kelps almost black and justifying their old name "Melanophyceae."

It is possible that some of the yellower Phaeophyceae, such as *Ascophyllum* or *Sargassum* [and sometimes *Fucus*, cf. Montfort (45, 48)] may be richer in other carotenoids, and show less activity in the blue (like *Dunaliella*). As far as this reviewer is aware, no action spectra have been taken with Dinoflagellates or Chrysophyceae; these algae contain quite different carotenoids, and should be investigated.

Red algae.—We now turn to perhaps the most remarkable pigment situation known. These algae have abundant chlorophyll-*a*, the closely related chlorophyll-*d* (unique to them) in rather low concentration [Manning & Strain (57)], and at least two carotenoids, β-carotene and lutein (xanthophyll). Absorption by all these is often as great as in green algae of comparable thickness. Yet these algae invariably contain high concentrations of the phycobilins even when, as in some *Porphyras* and in the fresh water *Batrachospermum*, the thallus appears almost green to the eye. These phycobilins effectively close the gap in the chlorophyll-carotenoid absorption spectra, being almost complementary to them, as pointed out earlier. Absorption is more nearly complete, therefore, than in any other plant: a single layer of *Porphyra* cells may absorb 70 to 80 per cent of the light throughout the visible spectrum (except around 620 mμ, where it falls below 60 per cent). How effective is the absorption?

Many physiologists have been attracted to this problem, because of the intrinsic challenge of the bright red thalli, and because some hope existed that a clear answer could be obtained to the question of accessory pigments, which here absorb so distinctly, and without great overlap into the chloro-

phyll zones. The answer was as remarkable as the color: chlorophyll was less active than phycoerythrin.

This result was clearly and elegantly obtained by Engelmann in the red algae *Callithamnion* and *Ceramium*. The indicating bacteria accumulated in the middle of the spectrum with a sharp peak (a veritable Matterhorn!) at 575 mμ in the green. There was a barely perceptible "foothill" in the red, and nothing but a valley in the blue. Clear participation of phycoerythrin was indicated; and the chlorophyll-carotenoid activity was so low as to be startling, —perhaps to cast doubt upon the method. It seems to have taken nearly 70 years for physiologists to concede that Engelmann was right, although most of the results obtained in the intervening years were completely consistent with his findings.

Actually, some six or eight workers, using over fifteen genera of red algae, came roughly or precisely to Engelmann's conclusion, von Richter (58) alone disagreeing. Ehrke (59), Montfort (45), and Schmidt (60), using the same methods as for the brown algae (see above), found enhanced photosynthesis in the middle of the spectrum (green) compared to red or blue: just opposite to the green and brown algae. Wurmser (61) and Klugh (62) employed the Osterhout-Haas pH indicator method (phenol red) to follow CO_2 utilization, and found the same difference between red and green algae: enhanced activity in the middle of the spectrum for red algae. Klugh's result was more striking than Wurmser's, probably because he used more effective filters.

Much better isolation of spectral regions was accomplished by Levring (50) who used combinations or "differences" of filters to give some 13 spectral bands of different width (some very broad), of which some 6 or 7 were fairly well separated. Eleven species of red algae agreed in showing oxygen evolution (by Winkler determination) in the middle of the spectrum (centered at 530–540 mμ) some two or three times that at either 430 or 670 mμ. There were also marked depressions between these three maxima. Levring's curves are necessarily "smoothed," because the broad spectral zones employed do not permit of great detail in the spectra. He did not take absorption spectra of the species he used, relying on already published data [Seybold & Weissweiler (63)]; there is, however, fairly good correspondence between the photosynthetic maxima and the absorption of phycoerythrin. Since absorption by phycoerythrin is not much greater than by chlorophyll in these algae, a considerably lower activity of chlorophyll is indicated; one half to one third that of phycoerythrin. The combined chlorophyll-carotenoid activity is in several cases even less than this.

Haxo & Blinks (64), by a polarographic (amperometric) method, had reported a photosynthetic rate in *Schyzimenia* nearly three times as great at the mercury green line (546 mμ) as in a red band (620 to 660 mμ), and six times as great as at the mercury blue line (435 mμ). This method was very quick and sensitive, hence, ideally adapted to detailed action spectra, taken

with monochromatic light of necessarily low intensity. Red algae were therefore studied in great detail (39) for comparison with *Ulva* and *Coilodesme* (mentioned above). The result with *Delesseria* is typical, and shown in Fig. 2*b*. Not only is the broad region of phycoerythrin absorption most effective in photosynthesis, but even the detailed absorption peaks (495, 540, and 565 mμ) have corresponding photosynthetic maxima. On the other hand, photosynthesis falls to almost a minimum at the chlorophyll maximum in the red (675 mμ) and even lower in the blue—at both of these less than a third that in the green. It must be emphasized that chlorophyll and carotenoid absorption are just about as great here as in *Ulva* or *Coilodesme;* yet the action spectra are almost reciprocal.

In other red algae with different phycobilins (or ratios), the action spectra closely parallel the absorption spectra of the phycobilins. Thus, *Porphyra naiadum*, an aberrant member of the Bangiales (with many morphological peculiarities which will probably remove it from its present genus), has a special "B-phycoerythrin" with only two absorption maxima instead of three: its action spectrum faithfully records this peculiarity, with a two-peaked photosynthetic curve! (Fig. 2*c*). It also has an increased rate at 620 mμ, corresponding to an increased phycocyanin content. This latter tendency is carried still farther in *P. perforata* where phycocyanin predominates; in this species, photosynthesis is highest at 620 to 650 mμ (Fig. 2*d*).

In the unicellular red alga *Porphyridium*, with a single-peaked "C-phycoerythrin," photosynthesis is maximal at this peak [Duysens (65, 66); Haxo & Norris (67)]. On the other hand, in all these cases photosynthesis is minimal or rapidly falling toward a minimum, at the very point where chlorophyll absorbs (675 mμ); and it is even lower in the blue, where carotenoids are also absorbing.

The action spectra, which are relative, have been confirmed and reinforced by determinations of absolute rates, and quantum efficiency [Yocum (40)]. These were done by several methods: Winkler, Warburg, and Fenn (volumetric) techniques were all employed. The Fenn method gave the values in

TABLE II

QUANTUM EFFICIENCIES OF SEVERAL MARINE ALGAE
(collected from nature—not cultured)
ϕ = molecules O_2 per absorbed quantum

Wave length (mμ)	436	500	560	620	675
Ulva (green alga)	0.06	0.07	0.08	0.07	0.07
Ilea (brown alga)	0.1	0.09	0.09	0.09	0.1
Porphyra Nereocystis (red alga)	0.02	0.06	0.07	0.068	0.04
P. naiadum (red)	0.03	0.04	0.07	0.055	0.04
Delesseria (red)	0.04	0.06	0.06	0.05	0.03

[after Yocum (40)]

Table II. Yocum's results indicate that chlorophyll activity is indeed depressed in the red algae. Whereas *Ulva* required about 13 to 15 quanta to release a molecule of O_2 and the brown alga only about 10 or 11, the red alga required 25 to 50 quanta in the regions of chlorophyll and carotenoid absorption. Only in the middle of the spectrum, absorbed by phycoerythrin, did the quantum requirement (15 to 16) compare with that of green and brown algae.

It must be concluded that red algae, in utilizing phycoerythrin to increase effective absorption in the medium wave lengths (green and yellow), have lost efficiency correspondingly at the ends of the spectrum (red and blue). Chlorophyll efficiency is down to a third or a half of that in green or brown algae, and carotenoids still lower. This seems a high price to pay for utilization of green light. Although the carotenoid situation is perhaps not so startling, one must ask how it is that some 50 to 60 per cent of the red algal chlorophyll-*a* is apparently inactive. There is nothing in its absorption spectrum or chromatographic behavior to suggest this fact; Manning & Strain (57), however, noted that red algae yield their chlorophyll-*a* very slowly to methyl alcohol or acetone. Is much of it bound to different proteins and so unavailable to the enzyme systems? Is chlorophyll-*d* an inadequate partner for its activity?

Is part of the chlorophyll-*a* so located topographically that it cannot pass its energy to the small active fraction although phycoerythrin apparently can? These are questions involving subtle differences in chlorophyll structure or conjugation, chloroplast topography, or possibly competition for enzyme systems which demand further study. One thing can be said, however: the situation is environmentally conditioned, to some degree for, under proper conditions, the chlorophyll can be activated. (See Adaptation, below.)

Blue-green algae.—Again Engelmann's results strongly indicated the participation of phycocyanin in the photosynthesis of *Oscillatoria*. The greatest oxygen evolution was in the orange with a maximum at 620 mμ, close to the absorption peak of phycocyanin. It was only 80 per cent of this value at the chlorophyll maximum, and down to 40 per cent in the short end of the spectrum (480 mμ).

Emerson & Lewis (68) confirmed the importance of phycocyanin in the unicellular blue-green alga, *Chroococcus*. The quantum efficiency at 615–620 mμ (where phycocyanin absorbs most of the light) was 0.08, almost as high as at 675–680 mμ (the chlorophyll maximum) where it was 0.088. Apparently light absorbed by the phycobilin pigment is used almost as efficiently as that absorbed by chlorophyll. On the other hand, carotenoid absorption was very ineffective. At 480 mμ photosynthesis fell to almost a quarter of the maximum, with a quantum efficiency of only 0.02. However, it rose again to almost 0.07 at 420 mμ which was somewhat higher than that calculated on the

basis of completely inactive carotenoids. Thus, we have a situation still different from the red algae; for although the phycobilin is active, so also is chlorophyll; only the carotenoids seem inactive, with perhaps only 20 per cent efficiency.

Chroococcus is not, however, completely typical of blue-green algae. Haxo & Blinks (39) reported marine *Oscillatoria* and *Anabaena* with action spectra peaked only at 620 mμ, falling away rapidly toward both chlorophyll and carotenoid regions (rather like Fig. 2d). This was confirmed by Duysens (66) in *Oscillatoria*. Haxo & Norris (67) have found a rather similar curve for *Phormidium ectocarpii* a blue-green alga which contains a high concentration of phycoerythrin, with a consequent shift of the maximum toward the green. All of these show low activity of chlorophyll and carotenoids. Apparently the blue-green algae are rather variable, and may differ considerably in nature and in culture. (See Chromatic Adaptation, below.)

Higher Plants

These are monotonously uniform in regard to the pigments in the photosynthetic organs (varied as they may be in flower and fruit). Strain (14) lists (in addition to chlorophylls-*a* and -*b*), β-carotene, lutein (=xanthophyll), isolutein, neoxanthin, flavoxanthin (*b* and *c*), zeaxanthin, and cryptoxanthin (the latter only in traces) from barley leaves. Essentially, the same were found in 24 other plants (herbs, shrubs and trees); some unidentified pigments occurred in conifers. Hey (69) has described eloxanthin, an isomer of flavoxanthin, from *Elodea* (which is well adapted for photosynthetic studies). These have rather similar absorption spectra, many with peaks around 420, 445, and 475 mμ (in ethanol), making it necessary to group them in any functional study. It would be difficult indeed to decide whether carotene was effective and lutein not; most workers would be content to decide whether the chlorophylls were effective and the carotenoids not!

Comparatively few studies of action spectra or detailed quantum efficiency like those in green, blue-green, or brown algae, have been made with the higher plants. (The action spectrum of photoperiodism is far better known.) This is understandable, not only for the reasons given above (monotony of pigments), but also because of a more difficult optical situation and because methods of measuring photosynthesis rapidly and over short periods, with small areas illuminated monochromatically, are not available for the higher plants. The best known studies are those of Hoover (70), Gabrielsen (71), and Burns (72, 73, 74).

Hoover used wheat seedlings, illuminated with light bands isolated by Christiansen filters, and found a two-peaked action spectrum (without great detail), on the whole, corresponding to total leaf absorption spectra [cf. Rabideau, French & Holt, (75)]. Since absorption in the blue end of the spectrum is at least half due to carotenoids, the probability is that some of the

carotenoids were absorbing light effectively, though perhaps not quite as effectively as chlorophyll, because the assimilation maximum in the blue was only about 85 per cent as high as in the red.

Gabrielsen (71) studied *Sinapis, Corylus,* and *Fraxinus.* He found that orange-red light was most effective, greenish-yellow next, and violet-blue least; in the order 1: 0.62; 0.37. This he ascribed to a lower absorption in the short end of the spectrum, but this is not in agreement with the absorption curves of various leaves taken by Rabideau *et al.* (75) in an Ulbricht sphere which takes account of scattering. All of these display about equal absorption (80 to 90 per cent) in the red and the blue-violet. Gabrielsen's results probably indicate inactive carotenoid absorption, just as in some of the algae. (As an example of a filter effect, Gabrielsen gives the photosynthetic rates of "red" varieties of *Corylus* and *Prunus;* these use blue-violet light more effectively than yellow-green, because the latter is absorbed by the anthocyanin.)

Burns (72, 73, 74) studied pine and spruce as well as wheat, finding relative photosynthesis and quantum efficiency of pine appreciably lower in the blue region of the spectrum. He concluded that there is inactive absorption in that region—presumably by carotenoids. (There were slight differences between pines grown in red and blue light.)

It is possible that very interesting results would be obtained with higher plants studied in spring and in autumn, when the pigment complex, and probably the whole photosynthetic mechanism, is rapidly changing.

CHROMATIC ADAPTATION

Studies of this problem go back as far as Gaidukov (76, 77) (who was inspired by Engelmann's results). Gaidukov found color changes in blue-green algae, resulting from exposure to different spectral regions. These were usually complementary to the color of the light used. Thus the algae became green in red light, purple in blue light. He also found that a ten-day exposure of the red alga *Porphyra* to intense red light caused it to become green. The latter effect, however, was possibly a bleaching of the other pigments, unmasking the chlorophyll, which was present already (such bleaching occurs in various red algae exposed to the sun at high intertidal zones). Gaidukov did not apparently measure photosynthesis in his adapted algae, and his work has been criticized by some, e.g., Sargent (78) as due to intensity effects, not color.

Harder (79) measured photosynthesis in chromatically adapted blue-green algae (*Phormidium*). He found that rates were in general higher in lights of complementary color to the algae; the adaptation was apparently photosynthetic as well. He used, however, such broad spectral regions (transmitted by dyes or copper salt solutions) that is is difficult to state what pigments were becoming adapted: chlorophyll, phycocyanin, or phycoerythrin. Absorption spectra of the algae were not taken.

Yocum (40) was the first to show that "functional" chromatic adaptation could occur; red algae could be adapted to use pigments which they already contained, without marked change in appearance or absorption spectrum. He found that *Porphyra Nereocystis*, when exposed for about a week to red light, very free of shorter wave lengths, began to grow faster; its action spectrum then indicated increased chlorophyll activity, and its quantum efficiency in red light approached that of a green or brown alga (0.07). That, in blue light, however, was not enhanced. If the plants were grown in intense blue light (436 mμ), they also became better able to use red light *but not blue*. Carotenoids were apparently not activated though chlorophyll was. No great change in absorption spectrum was produced though there was some change of color to the eye. No adaptation occurred in the dark nor, of course, in white light in which the plants had been grown in nature.

De-adaptation.—Yocum found that even a few hours of exposure to green light (546 mμ) rapidly de-adapted the plants, depressing their chlorophyll activity to its normal, "wild" level. This is doubtless the reason for the low chlorophyll efficiency of the natural algae, exposed as they are to high intensities of green in the sunlight. Presumably, it is absorption by the phycoerythrin which thus de-activates chlorophyll; the effect suggests a "competitive inhibition": when phycoerythrin is able to absorb light it monopolizes the photosynthetic mechanism which in red (or blue) light is slowly made available to chlorophyll. The mechanism of this competition or inhibition is not clear. It is not reciprocal, for the red adapted plants are still able to use their phycoerythrin. It is conceivably due to a photodynamic action of the phycoerythrin (e.g., splitting much of the chlorophyll from its proper enzymes). It must be emphasized again, however, that there is little or no bleaching, or alteration of absorption characteristics of the pigments; it is a change in their energetic or chemical coupling, by which much of the chlorophyll, previously active, is now unable to participate in photosynthesis. When it is understood, we shall probably also understand the nature of the inactive chlorophyll of red algae.

Adaptation has been observed in *Porphyra perforata* [Yocum (40)] and in *Porphyridium* [Blinks (13)]. The higher red algae (Rhodophyta) so far tested have not shown red adaptation, however. The blue-green algae should also be studied in view of their variable action spectrum.

MECHANISM OF PARTICIPATION

The reasons for inactive absorption are in some cases topographic. In other cases, one must postulate submicroscopic barriers or discontinuities— possibly at the molecular level (e.g., absence of conjugation with proper proteins). As just noted, this may apply to chlorophyll as well as to carotenoids. Regarding active "accessory" absorption, however, two choices exist. The accessory pigments may channel their absorbed energy entirely into chloro-

phyll (or an active fraction thereof); or they may have an entirely separate, parallel system of enzymes, able to carry on photosynthesis independently of chlorophyll. Most evidence points to the first alternative (a common pathway). Haxo & Blinks (39) found that red algal photosynthesis saturated at the same level for light absorbed by phycoerythrin and chlorophyll, although at about one-third the intensity in the former case. If saturation was first induced with red light, then addition of green light had no further effect, and vice versa. Poisons also altered both red and green light photosynthesis alike [Yocum (40)]. The time course (induction period) was also nearly the same, though green light tended to produce more of an "oxygen gush" than red. In red-adapted plants, there was a "transitional effect" during two or three minutes on alternate exposures to red and green light of equal intensity (and equal steady-state effect). There is apparently some residual "coupling and uncoupling" at work even during such brief exposures, probably of the same sort which adapts or de-adapts the plants completely on long exposure [Blinks (13)]. But this is only a fraction of the total rate.

Fluorescence.—The most conclusive evidence for a common pathway *via* chlorophyll is that of fluorescence. This is not surprising in the case of plants which use their chlorophyll efficiently. Dutton, Manning & Duggar (80) demonstrated that the chlorophyll of living diatoms fluoresced when illuminated by green light (absorbed chiefly by fucoxanthin) just as intensely as when excited by red light (absorbed by chlorophyll). This was confirmed by Wassink & Kersten (53). Since photosynthetic efficiency was also high in such light, the conclusion was that the accessory pigment passed its absorbed energy to chlorophyll with high efficiency. Part of this energy (0.1 per cent) was emitted as fluorescence in the long red light wave length, but most of it was utilized. French & Young (81) have shown the same situation in the red alga *Porphyridium.* Green light, absorbed by phycoerythrin, causes the fluorescence of chlorophyll much more efficiently than does blue light (absorbed by chlorophyll itself). Indeed, the "excitation spectrum" of chlorophyll fluorescence resembles the absorption spectrum of phycoerythrin, up to 550 mμ. The "accessory pigment" apparently excites chlorophyll fluorescence more effectively than does chlorophyll itself; on the other hand, absorption by chlorophyll and carotenoids does not seem to excite the fluorescence of phycoerythrin at all, even though the wave length relations are such that this reciprocal excitation might be expected.

French and Young also found that absorption of light by phycoerythrin excited a longer wave length fluorescence characteristic of phycocyanin (red, centered at 655 mμ). They concluded, as did Duysens (65, 66), that energy was transferred from phycoerythrin to phycocyanin, and probably from the latter to chlorophyll. If this is the necessary mediator then it is easier to see why chlorophyll cannot excite phycoerythrin fluorescence; phycocyanin's absorption lies at a longer wave length than phycoerythrin's.

Another indication that chlorophyll is excited by the other pigments as a necessary part of photosynthesis, comes from the measurement of the time course of fluorescence. Yocum (40) found, as did French and Young, that the "Kautsky effect," an outburst of fluorescence during the first moments of illumination, is restricted to chlorophyll fluorescence; the phycoerythrin fluorescence was steady. Since the fluorescence burst is accepted as having some relation to the induction period of photosynthesis [cf. Wassink (82)] it seems likely that the chlorophyll fluorescence of red algae indicates the active participation of that pigment, even when excited indirectly. It also seems to indicate that the large amount of "inactive" chlorophyll is not fluorescent; if it were, its fluorescence might swamp out the Kautsky burst of the active fraction. Study of the time course of fluorescence of red-adapted algae would be interesting in this connection; French made one measurement with such plants which indicates an increased chlorophyll fluorescence [Blinks (13)].

As a corollary to these findings, Duysens (65) reported a hitherto unknown, infra-red fluorescence of *Porphyra laciniata*, well beyond the chlorophyll-*a* peak at 690 mμ. He suggests that this might be due to the red algal pigment, chlorophyll-*d*, which has its absorption maximum (in solution) at about 686 mμ, with a diffuse fluorescence around 750 mμ [Manning & Strain (57)]. Duysens makes the intriguing suggestion that this fluorescence might represent a draining away of much of the energy from chlorophyll-*a*, thereby accounting for its low efficiency. If this is true there ought to be much less of such fluorescence in red-adapted algae. Also, the content of chlorophyll-*d* is remarkably variable amongst different species of red algae (57); algae rich in it (such as *Erythrophyllum*) might have very different action and fluorescence spectra from those poor in it (such as *Hymenena* or *Halosaccion*). Measurements with these would be desirable, as a test of Duysen's suggestion.

Mode of transfer of energy.—Accepting as most likely, the transfer of energy from accessory pigments to chlorophyll, we must ask how this can be accomplished. Arnold & Oppenheimer (83) have considered this question theoretically. Two possibilities, chance collisions between the molecules of pigments and fluorescence with reabsorption, seem too inefficient to agree with the good quantum yields observed in excitation by the accessory pigments. The most likely possibility is resonance or "internal conversion." For this to occur, the pigments must lie within a "wave zone" which is small compared to the wave lengths of light concerned. Calculations indicate that the distance between phycocyanin and chlorophyll molecules in *Chroococcus* was between 7 and 40 Å. (very small compared to the wave length of fluorescence) giving a strong likelihood that energy could be transferred by resonance with a minimum of loss (actually 1 to 2 per cent fluorescence loss was found in *Chroococcus*).

Since the pigments are probably still more closely crowded in the plastids of red and brown algae, internal conversion would seem further favored in those plants. Phycoerythrin has a very low fluorescence in living red algae, this increases greatly on injury or death, when the pigment diffuses into the cytoplasm. It has been suggested by Wassink (84) that the accessory pigments are attached to the same protein molecules as chlorophyll. If so the opportunity for energy transfer might be further enhanced. There is evidence for this in green and brown algae, but the phycobilin pigments seem to have separate proteins. We have so far not been able to prepare a complex of chlorophyll-phycoerythrin-protein that does not separate in electrophoresis [Blinks (13)]. On the other hand, there may be a loose complex between phycoerythrin and phycocyanin in some red algae. If, as seems likely, this is also linked with phosphate, there may be further implications as to energy transfer.

As a final comment, it may be noted that all the work so far discussed concerned the nonchlorophyllous pigments from the standpoint of light absorbers. But it has, from time to time, been suggested that they participate chemically, as oxidation-reduction systems. Dorough & Calvin (85) attempted to determine whether O^{18} derived from water enriched with this isotope might combine with carotenoids in photosynthesis, forming epoxide and furan structures (which might later release oxygen and regenerate carotenoids). Technical difficulties prevented a conclusive answer, but the authors consider the suggestion worth further investigation. If the idea is substantiated, a new function would be at hand for the widely distributed carotenoids.

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LOCALIZATION OF ENZYMES IN THE CELLS OF HIGHER PLANTS[1]

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Knowledge of the actual site of action of a specific enzyme is essential to an understanding of the physiological role of an enzyme in the economy of the cell or organism. Prior to 1950 only scattered references to this problem are found in the botanical literature. Much of the earlier work was based upon cytological observations rather than upon critical enzymatic studies; some of this literature has been reviewed by Weier & Stocking (1). Most of the work on intracellular localization of enzymes has been confined to studies on mammalian liver tissue, using the technique of differential centrifugation of crude tissue homogenates, first extensively used by Bensley & Hoerr (2). This literature has been discussed in a series of reviews of which those by Bradfield (3), Schneider & Hogeboom (4), and Dounce (5) may be of the greatest interest to the scientist wanting orientation in this field. The reviews of Weier & Stocking (1) and of Millerd & Bonner (6) deal specifically with the problems in plant cells.

This review will make no effort at complete coverage of the literature, and no attempt has been made to survey the Russian literature because of the difficulty of evaluating their briefly described methods. Emphasis will be placed primarily on a critical evaluation of the problems involved in the establishment of the site of action of an enzyme within a cell, followed by a more detailed summary of the enzymes localized within or on specific structures in the cells of higher plants. Enzymes considered to be soluble within the cell will be discussed only incidentally.

The data to be discussed has been obtained largely by the use of the method of differential centrifugation of cell-free homogenates, a technique which attempts to separate the components of the cells from each other on the basis of their size and density. Comparison of the results obtained by this procedure with those obtained by histochemical methods involving whole cells or tissues have been made by Dounce (5), Van Fleet (7), and Glick (8). Ideally, the two methods should arrive at the same conclusion, but the present histochemical methods appear to be limited to a very small number of enzymatic reactions. Although the validity of methods which involve homogenization of the cell are questioned by Newcomer and others

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[1] The survey of the literature pertaining to this review was completed in October, 1953.

(9), these methods, in spite of their imperfections and sources of error, have furnished most of the current information which is available, and in the present status of enzyme chemistry are often the only suitable experimental methods. While the results obtained from tissue homogenates and their fractionation should be accepted with caution, with proper controls and using a variety of tissues, this technique can add greatly to our knowledge of enzyme localization, even though the specific result cannot be used to calculate actual rates or to define mechanisms within the intact cell. Biological perfectionists will object to these studies as in an earlier period they objected to the use of tissue slices or parts of an organism because the results were not directly applicable to the organism as a whole. In an investigation of isolated cell components, one is studying the enzymatic potentiality of that isolated component in a specific environment and the results obtained are dependent upon the methods of preparation as well as the environment. However, when studies from many different laboratories using different species of plants or parts of plants and various methods of separation, reach similar or identical conclusions, these conclusions would appear to be fairly valid. Science advances by a series of successive approximations, and imperfect experiments are better than none at all.

In studies directed towards the determination of the localization of a specific enzyme on a definite cellular structure, at least three lines of description are essential. One, the isolated cellular structure must be described by a variety of modern cytological methods to identify the structure and to demonstrate the correspondence of the isolated component with a similar structure in the intact cell. Two, the isolated structure should be subjected to chemical analysis and description to further its identification. Three, the enzymatic reaction should be characterized unambiguously.

Cytological identification of the particle involved.—Any preparation of isolated cellular structures should be studied by standard cytological methods to determine the degree of heterogeneity. This heterogeneity may be merely one of size and shape of one particulate type, or it may actually involve particles of different chemical and enzymatic composition. The method of differential centrifugation applied to aqueous solutions can separate particles into different fractions, only if there is a difference in size or of density. In general, cell walls, starch grains, and unruptured nuclei or chloroplasts, can be separated from mitochondria or microsomes. Though this is a powerful tool, and is almost universally the initial method of separation, it is usually inadequate to give a homogeneous preparation of cellular particles. Uniformity of size may not be a decisive indicator of homogeneity; for instance, proplastids and grana may be in the same size range of mitochondria. The density of the suspending medium must not be ignored, or the fact that the particles may shrink or swell in a specific medium, because the gravitational field acts only on the difference in density between the particle and the suspending medium. A refinement of the centrifugal method is to layer the fraction on the top of media of different densities before centrifugation (10, 11). Another

possibility is that electrophoresis of particles may be adapted to isolated cellular components, taking advantage of differences in surface/charge ratio as pH varies to effect a separation of different particle types.

As a first approximation, the following average values of particle diameters (assuming spherical shapes) for some of the obvious cellular particulates from liver tissue (12) and pea cotyledons (13) are shown for comparison. The overlapping of the size ranges of mitochondria and plastids presents a considerable technical difficulty in the separation of these particles by differential centrifugation.

	Liver	Peas
Microsomes	0.06–0.15 μ	?
Mitochondria	1.3 μ	0.1–6.0 μ
Plastids	—	4–30 μ
Nuclei	50–100 μ	8–15 μ

In addition to the use of standard cytological stains, special optical methods are often valuable aids in the characterization of a particulate structure. For instance, the identification of leucoplasts by optical birefringence with polarized light of the contained starch grains may be used to distinguish leucoplasts from mitochrondria. The phase microscope has many advantages over the ordinary light microscope for observation of particles near the limit of resolution. The use of the electron microscope on ultra thin sections (0.05 μ) of fixed cells (14) and on isolated structures, may prove to be the most promising method to demonstrate the correspondence between an isolated structure and its homologue in the living cell. The correspondence of isolated liver mitochondria with those in intact cells has been adequately determined by Hogeboom, Schneider & Palade (15) and Harman (16). Similar identification is still lacking in plant material (see under mitochondria). Preer (17) has observed mitochondria within Paramecia under the phase contract microscope as well as the same structure after rupturing the cells in various media.

Chemical identification.—As certain chemical compounds may be characteristic of individual structures, chemical analyses can be instrumental in demonstrating that an isolated fraction consists, at least in part, of the specific structure. Obviously, isolated nuclei or chromosomes should be rich in desoxyribonucleic acids, and as McClendon (18) observed, nuclear contamination of chloroplast preparations could be assumed because of the presence of this acid. Chloroplasts are unique in containing chlorophyll, and mitochondria are usually characterized by ribosenucleic acids, although this component also appears in microsomes, chromosomes, nucleoli, and chloroplasts (4).

When pure preparations are difficult to obtain, the association of chemical structure with a particle can be a useful criterion of enzymatic localization. For instance, McClendon (19), in his preparation of a particulate fraction containing cytochrome oxidase from tobacco leaves, was able to show that

the activity did not follow the chlorophyll content, and though he never obtained active preparations entirely free of chlorophyll, he was able to conclude, with fair certainty, that the chloroplasts did not contain cytochrome oxidase. In contrast, Laties (20) in a study of a cyanide-resistant oxidase of spinach leaves, was able to show that the activity of the preparation was directly related to chlorophyll content, and presumably his oxidase was located within the chloroplast.

Chemical analysis may be the best method of distinguishing between proplastids and mitochondria, if there really are two particle types, for one might expect proplastids to contain protochlorophyll or chlorophyll plus carotenoids. Since it is difficult to be sure that the cytologists' definition of a structure is directly applicable to the isolated structure, a chemical definition of an isolated component may be preferable. For instance, microsomes are perhaps best defined at the present time by their high concentration of ribose nucleic acids in contrast to other cell particles.

The ratio of two or more chemical substances may be the most satisfactory method of identification. For example, Jagendorf (61) has found that the chlorophyll-protein ratio is a constant throughout his preparations, and Stern & Mirsky (27) have used the DNA-P protein ratio to show whether isolated nuclei have lost protein.

Through the workers isolating mitochondria, microsomes, and nuclei from animal sources have regularly included chemical analyses of the preparations, this has been the most neglected aspect of the studies of plant particles, except for the chloroplasts.

Enzymatic identification.—A particulate preparation may be characterized by its enzyme content; and this may serve to be its best description, However, in a particulate preparation several enzymes may be present, and it may be difficult to determine the actual course of the reaction. Ideally, one enzyme reaction should be analyzed at a time, and products should be identified and shown to be equal to the substrate disappearing.

When the enzyme may also be obtained in soluble form, it is far easier to isolate a single reaction. For example, the condensing enzyme is present on mung bean particles in association with many other enzymes, but this enzyme can readily be extracted from acetone powders of mung bean seedlings (21). With particulate preparations, single steps, or at least simplified reactions, may often be obtained by working in the absence of oxygen, by the addition of a specific inhibitor, or by withholding a coenzyme. However, many coenzymes are normally present in the particles and are not easily removed.

In spite of these difficulties, much has been learned concerning particulate enzymes. Standard biochemical procedures may be adapted to particulate enzyme preparations. While the direct determination of the disappearance of a substrate or appearance of a product as in the spectrophotometric determination of reduced pyridine nucleotides for dehydrogenase enzymes is probably preferred, such determinations are not always possible because

of interfering substances like pigments or the necessity of substrate quantities of coenzymes. Among the manometric measurements used (22) are those involving CO_2 uptake or evolution by either carboxylase systems or those resulting from changes in hydrogen ion concentration in bicarbonate media, and oxygen uptake using terminal oxidases or autoxidazable dyes. Dye reduction studies using tetrazolium salts or 2,6-dichlorophenolindophenol are quite sensitive (23) and easy to use for routine semi-quantitative assays. Considerable care must be taken to eliminate pH effects, and flavoprotein required to transfer the hydrogen to the dye should be added if this is a limiting factor. The dye method should not be considered specific for any one dehydrogenase, and nonpyridine nucleotide enzymes like succinic dehydrogenase are able to transfer the hydrogen to the dyes, presumably via a flavoprotein.

The development of the differential spectrophotometer by Chance (24) has added a powerful tool for the determination of the localization of enzymes and the rates of their activity *in situ*. By recording the change in optical density at specified wave lengths and measuring the disappearance of O_2 with an oxygen electrode, the activity of cytochrome-a_3 (cytochrome oxidase), cytochrome-c, cytochrome-b, and of the diphospho- and triphosphopyridine nucleotides have been followed.

The enzymatic activity of a particulate fraction should always be expressed in terms of the percentage of the total activity of the original homogenate as well as the specific activity or concentration of activity per unit of dry weight, protein, per plant, etc. As there is no best concentration unit, the practical one of the moment must suffice. For the special case of chloroplasts, the activity per gram or mole of chlorophyll is most useful.

Problems involved in the technique of differential centrifugation.—The process of breaking open a cell is undoubtedly a drastic one, and the possibility of artifacts must always be kept in mind. Such artifacts arising as a result of procedural methods have been discussed frequently (1, 3, 4, 5). Good examples of incorrect localization in a particulate structure due to artifacts are the precipitation of carbonic anhydrase with plastids when tannin is present in the tissues (25), and the association of catalase with plastids at a certain pH range (26). Enzymes extracted as soluble cytoplasmic constituents likewise may be procedural artifacts, being actually associated with particles within the cell at least in part. For example, evidence of the leaching of enzymes from otherwise "intact" nuclei has been shown by Stern & Mirsky (27), and the probability that dehydrogenases are absorbed on or in the nuclei. McClendon & Blinks (28) have also shown that chloroplasts lose the pigment phycoerythrin, even in a concentrated sucrose solution; however, the pigment is retained in a polyethylene glycol medium. Some of these artifacts are probably peculiar to plants, being due to the pH of the vacuolar contents which may be quite different from that of the surrounding cytoplasm.

Certain precautions against artifacts can be taken. The mildest form of

disintegration involves the use of a mortar and pestle and sand, or of a glass homogenizer, but these are not practical for tougher materials, and a Waring blendor must be used. This does not have to be run at high speed and the time and temperature can be closely controlled. The addition of buffers can control the pH, although the danger of clumping particles is enhanced in the presence of inorganic ions.

Since some of the particulate structures possess differentially permeable membranes and act like osmometers to some degree, the tonicity of the surrounding medium upon disruption is of great importance. Laties (29) has shown that both the tonicity of the medium during preparation of the particles and during the enzymatic incubation period are vital, and that these two factors are not necessarily the same even for one enzyme. It is also possible that the actual optimum conditions of extraction and incubation must be determined for each particle type, each enzyme, and for each plant source. A wide variety of aqueous extraction media have been used, ranging from water, inorganic salts (unbuffered and buffered), mannitol, sucrose, sorbitol, to high molecular weight polyethylene glycols (28). These have been recently supplemented by the use of nonaqueous media like cyclohexane-CCl₄ mixtures (30).

One difficulty in connection with tonicity is the determination of the best criterion of the optimum osmotic concentration. By microscopic observations, comparisons can be made of *in situ* and cell-free structures, but they are limited by the resolution of the microscope. A comparison of the dry weight with different extraction procedures can be a valuable tool in determining whether leaching has occurred, especially when microscopic observation gives no clue. For instance, a comparison of nuclei extracted in aqueous and nonaqueous media has shown that considerable protein is lost and subsequent alterations in the enzymatic distribution can be observed even though the nuclei appear intact microscopically (27). A third criterion of optimum osmotic concentration would be in the actual enzyme measurement, the higher rate presumably indicating a greater integrity of the original particle. A good example of this is the requirement for an externally added cytochrome-*c*, in a water-extracted mitochondrial fraction (13), in contrast to a sucrose-phosphate or mannitol medium (29, 31). This loss of a component can be due either to leaching or to an actual loss of catalytic activity (32). However, the assumption that higher activity denotes greater integrity of the particle may not be always valid, especially if "latent enzymes," ones which actually increase in activity upon disruption of a particulate structure, are widespread (32, 33).

Mitochondrial enzymes.—Perhaps the greatest problem concerning mitochondria today is the question of their definition. The various schools of thought concerning the morphology of these particulates have been amply reviewed by Newcomer (9). In general, the difficulties revolve around the problem of their morphological heterogeneity and whether this indicates more than one particle type, or whether they are all identical particles, some

of which remain as mitochondria and others (proplastids) differentiate into plastids. Newcomer (9) prefers to call all such particles mitochondria, even if they exhibit different biochemical functions.

Because of these difficulties, it seems best to define mitochondria in terms of their specific enzymatic activities, with chemical and morphological details as secondary factors. Such a definition will probably be more limited in contrast to the broader definition used by most plant cytologists, and would differentiate between proplastids and mitochondria because the enzyme complexes of these structures are entirely different. This does not eliminate the possibility that both particle types differentiate from a common smaller one. From a genetic point of view, it would account for separate cytoplasmic inheritance of the two forms.

Thus, the most appropriate definition applicable to both plant and animal tissue would be to define mitochondria as cellular particles associated with enzymes of the cytochrome system, the Krebs' cycle, fatty acid oxidation, and with oxidative phosphorylation. These lipo-protein and pentose nucleic acid-containing particles may vary in size from the limit of the light microscope, about $0.1\ \mu$ to $6.0\ \mu$ in diameter, and range in shape from spheres to long rods.

While liver mitochondria have been shown to have similar cytological aspects in tissues and in homogenates (15, 16), such positive identity has not been shown with homogenates of plant materials. In particular, the typical rodshaped mitochondria of intact tissues have not been satisfactorily duplicated in plant homogenates. However, there should be no question that the particulate preparations used by a variety of workers do include mitochondria (13, 29, 31, 34, 35) because they have been shown to contain at least some of the above-mentioned enzymes complexes. The question, however, is whether these preparations are pure mitochondrial preparations or contain other particulate types, and whether the lack of true rods in homogenates is merely a preparative problem. Millerd *et al.* (6, 31) identify their particles as mitochondria on the single basis of staining with Janus green B, but give no cytological detail concerning the variability of size and shape of the particles in their preparations. Davies (34) also states that his preparations of homogeneous spheres, about $1\ \mu$ in diameter, from pea seedlings stain with Janus green, but again no cytological detail is given. Stafford (13) likewise reported staining of particles with either oxidized or reduced Janus green B by observing the coloration of the centrifuged pellet of particles en masse, but was unable to see satisfactorily this stain in isolated particles with the light microscope. The use of Janus green B as a "specific" mitochondrial stain has frequently been questioned (9, 13), and the recent work of Lazarow and co-workers (36, 37, 38) throws further doubt on the use of this dye as a specific mitochondrial stain, especially in homogenates. The factors involved in the staining and reduction of this dye are quite complex, and are being studied by the above workers (36, 37, 38). The Nadi reagent and tetrazolium salts should likewise be used with great caution.

The specific enzymes studied by a variety of workers using relatively crude and perhaps heterogeneous particulate fractions containing mitochondria have been reviewed by Burris (39) and by Bonner & Millerd (6). The data are similar to that already reported for animal tissues. Demonstration of amino acid oxidation by plant mitochondria is still lacking. The low P/O ratios reported by Millerd and co-workers (31, 40) for oxidative phosphorylation are probably due to a loss of activity during preparation, as Laties (29) and Biale (41) have been able to report values approaching the theoretical ones. Originally the terminal enzyme for these particles, cytochrome oxidase, was hard to detect in preparations of green leaves, but Webster's survey shows that this enzyme is a part of the particulate enzyme complex of a wide variety of leaves and other plant organs (35). This still leaves the necessity of explaining the results of James (42) who postulates that ascorbic oxidase supersedes cytochrome oxidase as the terminal oxidase in mature tissues, especially roots. There is also a possible problem of the functioning of the Krebs' cycle in green leaves as indicated by the recent abstract of Brummond (43) although most of the individual enzymes could be demonstrated. All of the studies indicating the presence of a Krebs' cycle have used etiolated tissues (31, 34, 43, 44).

The dependence of the preparative treatment upon the association of hexokinase with mitochondria has been shown by Saltman (45) in wheat. Mitochondrial preparations made from wheat embryos individually dissected from the surrounding endosperm of the seeds contain 68 per cent of the total activity of the homogenate, while preparations from commercially prepared wheat germ contained only 33 per cent of the total activity.

In Table I we have brought together some of the literature concerning the localization of enzymes on plant structures. Not all of the papers referred to in this table have been discussed in the text.

A challenging recent contribution to mitochondrial knowledge is the further definition of the structural aspects through the use of the electron microscope. Most of this work has been done with animal tissues, but similar pictures are shown of sections of mesophyll tissue from plant leaves where both mitochondria and chloroplasts are visible (14). According to Palade (14) a mitochondrion consists of a double limiting membrane with a matrix that appears structureless at present levels of resolution. While there is a continuous internal cavity containing this matrix, there is a system of internal ridges or cristae which protrude from the inside of the membrane surface and appear to be folds of the internal mitochondrial membrane. His concept of a double membrane is consistent with the theory of Sjöstrand (46) who, likewise, has shown some excellent pictures of internal membranes within mammalian mitochondria. Palade postulates that structural mitochondrial enzymes like cytochrome oxidase may be localized on these membrane surfaces, while the easily solubilized ones (acid phosphatases) are a part of the homogeneous matrix. This concept of a particulate structure containing both

TABLE I

Enzyme	Mito-chondria	Chloro-plasts	Nuclei	Micro-somes	Surfaces
Cytochrome oxidase	13, 19, 35, 51				86
Ascorbic oxidase					89, 90, 92
Polyphenol oxidase	19, 61				
Cyanide insensitive oxidase		20			
Aldolase			30		
Isomerase			30		
DPN glyceraldehyde dehy.			30		
Enolase			30		
Pyruvate kinase			30		
Hexokinase	45				
Succinic oxidase or dehy.	13, 23, 29, 31, 34, 40, 41, 43, 44, 52				
α-ketoglutaric oxidase or dehy.	23, 29, 31, 34, 40, 41, 43, 44				
Malic oxidase or dehy.	29, 31, 34, 40, 41, 43, 44				
Pyruvate	29, 31, 34, 40, 41, 43, 54				
Oxalacetic oxidase	29, 31, 34, 40, 41, 43, 44				
Citric oxidase	29, 31, 34, 40, 41 43, 44				
Fumarase	29, 31, 34, 40, 41, 43, 44				
Oxidative phosphory-lation	29, 31, 40, 41, 44, 53				
Condensing enzyme	21, 55				
Acetate activating enzyme	55				
Co A	21				
Catalase	61				
Glutamine synthesis	56				
Glutathione synthesis	57				
Fatty acid oxidation				80, 81	
Hill reaction-photore-duction coupled enz.		62, 68, 69, 70, 71, 72			
CO₂ fixing into P-gly-cerate		73			
Cytochrome-f		74			
Unknown pigment		75			
Lecithinase		76			
Glycolic oxidase		77			
Phosphorylase		64			
Phosphatase					86
Sucrose phosphorylase					84, 85
Pectinesterase					91

"soluble" and structural enzymes is an interesting one, and may be applied to nuclei and chloroplasts as well (see below).

Practically no chemical analyses of plant mitochondria have been made, except for those reported by Stafford (13) who analyzed a possibly impure mitochondrial fraction for ribose nucleic acid (RNA) protein and total lipids. The value of <1 per cent of the dry weight as RNA 30 to 40 per cent protein and 25 to 38 per cent lipid was similar to that for liver mitochondria (47, 48), but the 20 to 30 per cent unidentified material is still unknown.

Another challenging field which may be facilitated by the above structural picture and the electron microscope is the one of the origin of mitochondria and their relationship to plastids and microsomes. The morphological heterogeneity and the restriction of staining reactions like Janus green and the Nadi reagent to only certain of the particulate structures in plant cells has been observed in leaves (49, 50). On the basis of a controlled Nadi staining technique, Perner (50) reports in epidermal leaf tissues that certain particles stain while others do not. He prefers to call the stained particles, presumably containing cytochrome oxidase, microsomes, and the unstained ones chondriosomes (mitochondria). This would be in contrast to other workers, but his microsomes are visible with the light microscope and his actual pictures show particles similar to those reported by Stafford (13) and McClendon (18) in tissue homogenates. Therefore, the discrepancy is probably one of definition rather than in enzyme localization.

Plastid enzymes.—The enzymes immediately involved in photosynthesis are presumably localized in the chloroplasts. The work concerning the present status of the enzymes involved in the Hill reaction and CO_2 fixation has recently been reviewed by Brown & Frenkel (58) and Hill & Hartree (59). The chloroplast fractions used in these studies have been described as containing whole chloroplasts and/or grana and plastid fragments. The purity of these fractions has never been established and they probably are contaminated with nuclear and mitochondrial particles (18, 19, 60, 61). Vishniac (62) reports the solubilization of some of the components of the Hill reaction and coupled enzyme systems in which whole chloroplasts or grana were substituted by a methanol extract of chlorophyll and an extract from an acetone powder of leaves. Although this may not be a path of reduction *in vivo*, it does represent the first solubilization of such a light-induced reaction.

Earlier work, reviewed by Weier & Stocking (1) attributed many enzymatic activities to plastids. Many of these are not considered valid by a number of workers, primarily because of contamination and ill-defined fractions (1, 19, 60). The problem of phosphorylase is worth further discussion. It has long been a well-established fact that starch grains arise inside plastids. Therefore, some of the final steps must be localized within the plastid membrane. The homogenate technique, however, using both water and sucrose media, shows that phosphorylase is not associated with any particulate fraction in seedlings, but is soluble (13). Stocking (63) arrives at the same conclusion using both homogenate and histochemical methods.

Paech & Krech (64), on the other hand, consider that this work is not valid and interpret their own histochemical data as showing that phosphorylase is located only within plastids. The assumption, however, that incubation of tissues with glucose-1-phosphate and detection of the end result, starch, with iodine is a valid enzymatic test for phosphorylase is not necessarily true. Glucose-1-phosphate may not enter the cell as such, and the final production of an iodine-staining starch grain involves a series of enzymatic activities besides that of phosphorylase, namely, the various branching enzymes (65). Phosphorylase itself produces only short linear chains involving 1,6-glucosidic linkages. If phosphorylase is truly a soluble enzyme, some explanation of how the products get into the plastid or what other enzyme system is substituted still needs to be explained. Stocking (63) suggested the possibility of a transglucosidase system, but this has not yet been demonstrated in higher plants. The other possibility is whether relatively short amylose or polyglucose chains could actually diffuse through the plastid membrane to be incorporated into the starch molecule. The discrepancies between Stocking's and Paech's histochemical techniques still need clarification. Recent work using isotopic glucose and fructose may further elucidate the starch and sucrose forming mechanism (66). The enzymatic relationships between chloroplasts and leucoplasts is likewise a problem that needs exploration.

The claims (1) that cytochrome oxidase occurs in chloroplasts have been shown by McClendon (19) to be due to the contamination of the plastid fraction by nonplastid particles, probably mitochondria. Jagendorf & Wildman (61) have similarly shown that the nonsoluble catalase (about one-half of the total) of tobacco leaves is on nonchlorophyll-containing particles, again, probably mitochondria, while Hagen & Jones (26) have studied the effects of pH on catalase localization.

The chemical analysis of chloroplasts is quite well documented (1), but carotenoids as well as chlorophyll might be used advantageously as a criterion of localization. In terms of total protein, nucleic acid, and lipid content, plastids do not differ radically from mitochondria.

Electron microscope studies are adding to our knowledge of chloroplasts. In particular, the work of Wolken & Schuertz (67) on the chloroplasts of Euglena might be noted. This work lends support to the lamellar structure of chloroplasts or grana postulated by prior workers in which lipoprotein and an aqueous protein layer are separated by a dense layer of chlorophyll. The average Euglena chloroplast (comparable with a single granum) is about 1μ in diameter and 6.5μ in length, and is made up of approximately 21 regularly oriented lamellae. These lamellae, or dense bands, are about 242 Å thick and the interlamellar spaces are 374 Å thick. Palade (14) shows pictures of chloroplasts of tobacco containing grana with similar lamellar structure.

Nuclear enzymes.—Cytological and chemical aspects of nuclei sufficient for identification purposes are well established and will not be further discussed (78). The enzymatic pattern associated with animal nuclei has been studied by numerous workers (30, 79), and while a large number of enzymes have

been found to be associated in part with isolated nuclei, they represent only a small percentage of the activity of the whole homogenate, and are present in a concentration on a dry weight or protein basis that is far less than that of other cellular sites. There is one main exception to this general pattern, i.e., alkaline phosphatase (79) where the concentration is higher in the nucleus, but the percentage of the total is still very small. The danger of contamination is very great when the activity represents such a small fraction of the total (4). As most of the nuclei studied represent only about 8 to 10 per cent of the total cellular volume, this small percentage is not surprising unless a particular enzyme were peculiar to nuclei.

Because of the difficulty of isolating whole nuclei from plant material containing mature cell walls which necessitates fairly strong disruptive forces to break open the cells, there have been practically no published reports on isolated plant nuclei. The recent paper of Stern & Mirksy (30) is of great interest because they combined the use of nonaqueous media for extraction and a source of nuclei from wheat germ in which the nuclear to cell volume is unusually large. Their nuclear fraction was not absolutely pure, but their results indicate that certain of the glycolytic enzymes generally considered "soluble" are present in approximately equal concentrations in terms of nitrogen and represent an unusually large percentage of the original total activity (50 to 80 per cent). They did not characterize their remaining fractions in detail, referring only to heavy and light cytoplasmic parts. Their method consisted of extraction in a cyclohexane-CCl$_4$ mixture followed by centrifugation and then testing in aqueous media for aldolase, glyceraldehyde dehydrogenase, enolase, and pyruvate kinase. This method is, unfortunately, limited to enzymes not inactivated by such organic media, and lipid-containing structures like mitochondria may be completely destroyed, though no mention of this is made in their paper. They report no negative enzyme analyses, and do not show any comparative data with water-containing media using the same wheat germ. They have ruled out nonspecific adsorption of enzymes by nuclei under these conditions by showing that amylase (another so-called soluble enzyme) is not found associated with nuclei under the identical conditions used for the above glycolytic enzymes.

These results are of great interest because of the long-known removal of protein from nuclei isolated from water media. Recently, using animal tissues, they have shown that certain "soluble" enzymes are easily washed out of nuclei from some sources (27). Schneider (32) does not think this leaching of soluble enzymes from nuclei in aqueous media is necessarily valid, but he does not explain his reasons in any detail. Further work needs to be done to see how widespread is this condition of high nuclear glycolytic activity. Wheat germ is an embryonic tissue ready for active growth upon germination. It is possible that more mature tissues might not have the same distribution. While these enzymes have not been widely studied in other tissues using nonaqueous media, the authors feel that the leaching condition may be widespread, as considerable protein is known to be removed in aqueous

media. Stern and Mirksy discuss the significance of their findings in terms of the source of energy for nuclear metabolism.

This investigation opens up the question of whether structures like nuclei, and possibly mitochondria and plastids, contain two forms of enzymes. The one type is tightly bound to structural components and will not leach out in aqueous media. Only the "proper" osmotic concentration is necessary to prevent disruption. Alkaline phosphatase, associated with nuclei in high concentration, may represent this type. The others are "soluble" within the nuclear membrane and actually exist in the same state as they would outside in the cytoplasm. These enzymes are easily leached out in aqueous media, and would include the glycolytic enzymes studied by Stern and Mirsky. Such an explanation could account for a loss of phosphorylase by isolated plastids.

Microsomes.—This particulate fraction is even less well-defined than the mitochondrial fraction. There is no clear-cut enzymatic pattern and their submicroscopic size (less than 0.1 μ) and high nucleic acid content seem to be the only satisfactory criterion of identification (4). There is also considerable disagreement as to whether microsomes are disintegrated mitochondria, undifferentiated mitochondria, or represent distinct particles with different metabolic functions from that of mitochondria (4).

The reports of possible microsome fractions isolated from higher plants are meagre. The presence of particles smaller than a possible mitochondrial fraction and richer in pentose nucleic acid was reported by Stafford (13), but no positive enzymatic characterization was made.

Stumpf and co-workers (80, 81) have recently isolated a possible microsome fraction as far as size is concerned (about 20 mμ in diameter) which is associated with a fatty acid oxidation system that is not the same as that reported for liver mitochondria (12). Presumably, plant mitochondria will be found to contain also a complete fatty acid system such as is found in liver mitochondria. Particles about 20 to 37 mμ have been isolated from aqueous extracts of bean root cells and characterized by electron microscope pictures (82). These disappear upon treatment with trypsin, but not with pepsin or ribonuclease.

Surface localized enzymes.—There is a large body of physiological literature which is best interpreted on the assumption that certain enzymes are localized at the cell surface. One need only recall the absorption of glucose against a concentration gradient in kidney tubule or by the intestinal mucosa, and the inhibition of such absorption by phloridzin (83). Dormer & Street (84) and Street & Lowe (85) have shown that tomato roots grown in culture grow far more readily on sucrose than on an equal molar mixture of glucose and fructose, and that the growth of such roots is inhibited by phloridzin. They postulate that a sucrose phosphorylase is located in the cell surface and, though sucrose phosphorylase is still undemonstrated in higher plants, the indirect evidence is strongly suggestive that enzymatic mechanisms in the cell surface are involved in sugar absorption by roots.

Rothstein & Meier (86) have shown that yeast hydrolyzes added adenosine triphosphate and other phosphate esters, with the products of the reaction appearing only in the external solution and not in the cells. Again, the evidence is best interpreted on the assumption that these phosphatases are bound in the cell surface.

Except for indirect evidence by Waygood (89), ascorbic oxidase has been considered a soluble enzyme (13). Newcomb (90), however, has found that ascorbic oxidase was almost completely associated with the cell wall material of tobacco stem pith cultures which had been ground and extracted under mild conditions. Newcomb established that whole cells, nuclei, plastids, and mitochondria were not present in the cell wall fraction, and that harsher treatment solubilized the ascorbic oxidase. His experiments could not determine the actual site of activity on the surface; for example, one may suppose that it occurs on the cytoplasmic surface interpenetrating the cell wall.

Newcomb's method may not have a general application because of the difficulty of breaking the cells without solubilizing the surface-localized enzymes. One may suspect that a considerable number of enzymes involved in cell wall metabolism may be located in the surface, such as pectinesterase which has been recently reported (91). Perhaps adequate histochemical methods may be designed, or the indirect methods used by Rothstein and colleagues (86, 87, 88) and others may have to be depended upon.

Mandels (92) has found in the spores of the fungus *Myrothecium* a new ascorbic acid oxidase (not a copper protein) which his evidence indicates is on the spore surface.

Conclusion.—The challenging advances due to the use of nonaqueous media to detect easily-solubilized enzymes and to indirect and direct methods of identifying surface-localized enzymes should afford a strong impetus to further research in the field of enzyme localization. New methods, both for extraction and measurement, need to be found. Developmental studies of the cytological, chemical, and enzymatic aspects of cellular particles are necessary in order to determine relationships between structures like microsomes, mitochondria, and plastids, and in order to compare the localization during growth, development, and maturity.

One approach not yet mentioned, but which has proved to be a valuable biochemical tool, is the use of mutants in order to establish patterns too difficult to assess in the nonmutant organism. The work of Ephrussi (93) and Hirsch (94) in which the absence of certain oxidative enzymes is being studied in terms of particulate structure and enzyme localization, is an excellent case in point. The approach by Lwoff (95) in breeding chloroplast-free organisms which still contain mitochondria should be further investigated in terms of plastid-mitochondria relationships. Likewise, the mutants of higher plants studied by Woods & DuBuy (96) would serve as useful tools for the determination of enzyme localization.

If certain enzymes are not associated with known particulate structures and should validly be called soluble, just what is their relationship to par-

ticulate structures and to the protoplasmic components making up the ground cytoplasm of the cell? Schneider (32) comments on the relationship between the Krebs' cycle enzymes associated with mitochondria and the glycolytic enzymes of the surrounding cytoplasm in reference to the diffusion of intermediates produced by the one system and acted upon by the other. He feels that diffusion might not be adequate to account for efficient interplay between the two systems, and discusses theories concerning the possibility of mitochondrial movement and the possibility of enzymatic differences between particles now all called mitochondria.

The question of the validity of diffusion to account for intratransport of metabolic intermediates is worthy of further evaluation.

The most powerful method of following the activity of a specific enzyme within a living cell is that of differential spectrophotometer as developed by Chance (97). This method makes it possible to correlate the state of oxidation or reduction of a particular enzyme with the time when a substrate such as oxygen reaches zero tension. To date, it has not been useful in localizing an enzyme on a particular cellular structure unless that structure is first isolated from the cell. Similar methods have been developed by Lundegårdh (98 to 101) who has applied it specifically to the wheat root. Lundegårdh has correlated the state of oxidation of the cytochromes to the absorption of salts against a concentration gradient. To the authors, Lundegårdh makes an excellent case for the participation of the cytochromes system in salt absorption but he interprets his results to mean that the cytochromes are localized in the cellular surface. Recognizing the beauty of Lundegårdh's experiments, the authors are not convinced that his evidence establishes the site of these enzymes in the cell.

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THE PHYSIOLOGY OF PLANT TUMORS[1]

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Crown gall has throughout the past half century been commonly regarded as the experimental model in the field of plant oncology. The literature dealing with this disease is far more extensive than that of any other plant neoplasm. The present discussion will therefore be concerned for the most part with a consideration of the inception and development of the crown gall tumor. The general literature dealing with crown gall was critically reviewed in 1935 by Riker & Berge (172), and again in 1951 by de Ropp (56). Numerous, more limited discussions of this disease have appeared in the past two decades (22, 30, 31, 44, 67, 69, 70, 124, 154, 174, 177, 203, 206, 208, 226, 227). A list of more than 500 references to crown gall has been compiled by Elliott in the first two editions of her book "Manual of Bacterial Plant Pathogens" (63, 64).

The much larger problem of plant overgrowths generally has recently been dealt with by White (227). Manil (149) has discussed the pathological aspects of plant tumors caused by fungi, bacteria, viruses, hybridization, and physical and chemical factors. Various phases of this problem were also considered in No. 6 of the Brookhaven Symposia in Biology (33). Tumors of viral etiology have recently been discussed in detail by Black (8, 9, 10).

The Development of the Crown Gall Problem

That the fundamental aspects of crown gall have been studied so intensively during the past 50 years is doubtless due in large part to the early and profound influence that Erwin F. Smith exerted in the field of comparative oncology. The finding by Smith & Townsend in 1907 (186) of a specific bacterium as the causal agent of this plant neoplasm appeared significant to pathologists generally. At the time of this discovery no animal tumor had yet been produced experimentally. In an extensive and now classical series of papers (189 to 196, 198 to 201) covering a period of 20 years, Smith emphasized similarities between crown gall and animal cancer. Jensen (88) concurred with Smith in the belief that crown gall is comparable to malignant animal tumors. Jensen applied to plant tumors the grafting techniques that he had so successfully used in his investigations on animal tumor grafts. On the basis of rather limited studies, Jensen concluded that the plant tumor cells with which he worked were capable of autonomous development in the absence of any recognizable organism. Smith, nevertheless, remained, throughout his active scientific career, a staunch proponent of the parasitic

[1] The survey of the literature pertaining to this review was concluded in September, 1953.

theory not only of crown gall but of animal cancer as well. He stated (192 p. 256)

> The cell itself is not the parasite as Jensen thought, because we have proved these tumors to be due to a specific microorganism, a feeble, intracellular schizomycetous parasite which has no power to kill the cells but only the power to set them growing. Therefore, the "cell is the parasite" only in the sense that it is urged on by a schizomycete.

This concept was reflected in the thinking of later investigators and has even quite recently been repeatedly expressed in the literature. Although not always agreeing with Smith on the position of the bacteria in relation to the host cells (3, 86, 87, 167, 181), there appeared to be complete agreement among these workers that the crown gall tumor was a bacterial hyperplasia, the continued development of which was dependent upon continued stimulation by the inciting bacterium.

Although Smith regarded crown gall as a parasitic disease, he nevertheless clearly recognized the need for characterizing the physiological factor or factors associated with the bacterium and responsible for the pathological development of the cells. The stimulus responsible for abnormal cell division was at first believed by Smith (192) to be a bacterial endotoxin which acted on the cell nucleus. This toxin was thought to be liberated only after some of the bacteria within the cells had died. Attempts to secure cell stimulation with dead bacteria (13, 89, 213) or bacterial lysates (156) have repeatedly failed. In 1920 Smith showed that ammonia, acetic acid, and formic acid, three compounds elaborated by the crown gall bacterium in culture, were capable of effecting a limited proliferation when applied to cells of certain plant species. The production of ammonia by the crown gall bacterium seemed particularly significant to him (195, 197). He believed that the slow and continuous liberation of small quantities of this compound by bacteria imprisoned within cells was one of the important causes of abnormal cell proliferation. Subsequent studies (228) showed, however, that similar results were obtained with a nonpathogenic culture, indicating, according to Riker & Berge (172 p. 323) ". . . no direct correlation between ammonia production and pathogenicity as suggested by Smith" (195). The production of ammonia by most if not all of eight distinct species of gall-producing organisms was nevertheless considered noteworthy by Riker, Spoerl & Gutsche (175).

In 1927 Bechhold & L. Smith (2) isolated a thermostable colloidal substance from a culture filtrate which was purported to have cell-stimulating properties comparable to that of the crown gall bacterium. The results of these workers could not be confirmed despite repeated efforts (89, 213). Similarly, Boivin and collaborators (14) described impressive overgrowths that they claimed to have obtained by treating sunflower plants with the endoantigenic fraction of virulent crown gall bacteria. Rybak (182) has recently reported that neither he, Braun, nor Boivin himself could successfully repeat that work. Other chemical fractions isolated from the bacteria (71, 126, 218, 219, 220) when applied to plants produced only limited

cellular proliferation or no proliferation at all. The carcinogenic hydrocarbons (5, 99, 100, 121, 123, 125, 127 to 133) that have proven so effective when applied to animal cells have, with a few possible exceptions (99, 127, 128), been ineffective in inducing the cancerous change in plant cells. The reported exceptions merit further study. Since, however, some of the most powerful carcinogenic hydrocarbons bear a structural relationship to the sex hormones and certain other sterols found in the animal body, it might be more profitable to study the effects of compounds that bear a similar relationship to the plant hormones. A promising start in this direction has already been made by Gautheret (65), Limasset & Gautheret (134), Morel (153), and Camus & Gautheret (39).

Numerous chemical compounds of the most diverse type had been shown by 1935 (172) to have a limited stimulatory effect on the growth of plant cells. Levine (121) reviewed the literature dealing with this subject and concluded that in no case did the materials tested induce overgrowths that approached in size the large tumors initiated by the crown gall bacterium. Riker & Berge (172) agreed with this conclusion but suggested that the difficulties encountered may have been due to the experimental methods used. It was not possible at that time to introduce these substances into plants slowly and over long periods of time as had been postulated for the mode of the bacterial action. There was, then, a need for a carrier of chemicals that would permit the slow diffusion in sublethal doses of active materials. This need was satisfied with the introduction of lanolin for this purpose by Laibach (108). It was soon found (109, 110) that indoleacetic acid and related compounds were capable of inducing overgrowths of considerable size when applied in lanolin to certain plant tissues. Using these procedures Brown & Gardner (36) demonstrated that dried ether extracts of suitable culture fluids in which the crown gall bacterium had grown gave rise to large overgrowths when applied to bean plants. These appeared, superficially at least, to be similar to tumors initiated by the crown gall bacterium. The findings of these investigators were soon confirmed and extended. The biologically active material gave a positive Solkowski test (135) and was considered to be indoleacetic acid (6). Naphthaleneacetic acid, indolebutyric acid (75), as well as tryptophan (103) were found to exert stimulatory effects similar to those produced by indoleacetic acid. Kraus, Brown, & Hamner (102) and Levine (128) studied the histological effects of indoleacetic acid on plant tissues and concluded that they resembled in many respects the histological pattern found in crown gall tumor tissue. Link, Wilcox, & Link (136) made a comparative study of the effects of the crown gall bacterium, indoleacetic acid, active extracts of the culture filtrate, and wounding on bean and tomato plants. These workers concluded that galls are possibly incited by the crown gall bacterium through indoleacetic acid in conjunction with other chemical agents. Symptoms (47, 143, 161) (i.e., epinasty of petioles, initiation of adventitious roots, stimulation of cambium, inhibition of lateral buds, delayed abscission of senescent leaves, etc.) were described on certain plant species in-

oculated with virulent crown gall bacteria that were similar to those induced by indoleacetic acid. Plants inoculated with attenuated cultures showed these host responses to a far lesser extent or not at all. Cells of incipient galls initiated by an attenuated culture could be greatly stimulated by tumors initiated by a virulent culture (143). It was demonstrated further (137, 138) that the concentration of auxin was greater in tumor tissue than in an equal weight of normal tissue. In 1938 Dame (43) reported that, while indoleacetic acid is produced in culture by virulent crown gall bacteria, avirulent organisms of the same species produced little or none of this growth substance. As a result of these studies it appeared that the chemical cause of crown gall had been found.

Several lines of evidence were soon presented, however, which raised considerable doubt as to whether that interpretation was correct. It was indicated for example: (a) that the host range of indoleacetic acid effectiveness was different from that of the crown gall bacterium, i.e., some plants that responded to the crown gall organism did not respond to indoleacetic acid (143); (b) no correlation was found to exist between the ability of crown gall bacteria to elaborate indoleacetic acid and their ability to induce tumors (143); (c) not only crown gall bacteria but also related nongall-forming organisms produce growth substances (144); (d) incipient tumors initiated by an attenuated culture could not be stimulated with indoleacetic acid (143); (e) differences were found in amounts of auxin diffusing from stems bearing galls and control stems (143). No significant differences were found in auxin content between inoculated and control tissues 1, 4, 8, and 16 days after inoculation when comparisons were made on a total nitrogen basis instead of on a fresh or dry weight basis as had previously been done (173). Comparisons on a total nitrogen basis seeemed more reliable to Riker and collaborators (152) because it more accurately measured the living material. Despite this criticism recent studies (78, 104, 105, 137) have shown that crown gall tumor tissue contains considerably more auxin than does normal tissue.

It was at this stage in the development of the crown gall problem that evidence of several distinct types appeared which demonstrated unequivocally that normal plant cells may be irreversibly altered to tumor cells in short periods of time by the inciting bacterium of this disease (19, 20, 45, 223, 224). Upon completion of the cellular alteration the proliferation of the altered cells became an automatic process that was entirely independent of the inciting bacteria. Tumor cells of this type are characterized by their rapid powers of proliferation and their very limited powers of differentiation and organization. Fragments of bacteria-free tumor tissue but not thoroughly ground-up tumor cells, when implanted into healthy hosts, developed again into large overgrowths comparable to those originally initiated by the bacteria. Since these characteristics of rapid and autonomous growth have now been maintained unaltered by crown gall cells for more than ten years, it has been concluded that crown gall tumor cells are permanently altered cells that reproduce true to type and against the growth of which there is no control mechanism in the host.

The establishment of the fact that crown gall was a truly independent growth necessitated a reëxamination of interpretations given certain earlier findings. Although, as indicated previously, Locke and collaborators (143) had been unsuccessful in stimulating incipient growths initiated in tomato plants by an attenuated culture with indoleacetic acid, other workers (18, 212) reported that certain related compounds were highly effective in this respect. When certain of these growth substances were applied in lanolin above such incipient growths, the latter expanded rapidly and were comparable both in size and rate of development to tumors initiated by a highly virulent culture. The virulence of the attenuated culture was shown not to have increased as a result of exposure to the growth substances (18). The cells of such artificially stimulated overgrowths were transplantable through at least three transplant generations. Normal plant cells stimulated by these growth substances, on the other hand, fused with the host but did not develop into tumorous overgrowths when grafted to healthy plants. On the basis of this evidence it was concluded (18) that both virulent and attenuated cultures alter normal cells to tumor cells but perhaps to different degrees. In addition, a growth substance is required for full tumor development in cells altered by an attenuated culture, while cells transformed by a virulent culture generate an abundance of their own growth substance. This work also emphasized the need for recognizing a distinction between those factors that render cells neoplastic and those that affect their subsequent behavior. It was suggested (18) that tumor formation takes place in essentially two distinct phases. In the first phase normal cells are changed to tumor cells. The second phase, according to this concept, involves those factors concerned with the continued abnormal growth of the altered cells once the cellular change has been consummated. The virulent bacteria accomplished both phases, while the attenuated culture brought about essentially only the first phase. Here was an attempt then to delimit the etiological and physiological processes involved in the inception and in the development of crown gall tumors. Recently Klein & Link (96) have further subdivided these phases.

THE INCEPTION PERIOD

After it became apparent that crown gall bacteria were capable of inducing a profound and permanent change in the character of plant cells, attempts were made to determine the period of time necessary for the bacteria to accomplish the cellular alteration. By subjecting the host-parasite complex to a thermal treatment of 46°–47°C. it was found possible (19, 21) to kill crown gall bacteria at any desired time after they had established themselves in the host without affecting the capacity of the host tissues to respond with tumor formation to any cellular alteration that had occurred prior to the time the inciting organisms were destroyed. With the use of this method it was shown that normal plant cells may be converted into tumor cells as early as 34 to 36 hr. after bacteria are introduced into a suitable host. Tumors initiated in this early period grew very slowly and remained small. Some,

however, continued to develop and after an eight-month period reached moderate size (26). When, on the other hand, the bacteria were permitted to act on the host cells for 3 to 4 days before being destroyed, the resulting tumors were comparable both in size and rate of development to those found in inoculated but unheated control plants. Bacteria-free tumor cells isolated from overgrowths initiated in 36 hr. grew slowly on a culture medium when compared with the growth of cells altered in a 4-day period. This finding led to the suggestion that the transformation of normal cell to tumor cell takes place gradually, leading in a 3 to 4 day period to an autonomous, rapidly growing type of tumor cell. In the light of these findings it is clear that the bacteria or some factor from the bacteria pass to the host cells and bring about in short periods of time a complete and permanent change in the subsequent behavior of affected cells. This biologically active principle will hereafter be referred to in this discussion as the tumor-inducing principle (TIP). That plant tissues can be freed of crown gall bacteria by thermal treatment has been confirmed and extended to show that, in addition to high temperature, a relative humidity of 60 per cent or more is essential to accomplish destruction of the bacteria (210).

A second method particularly suited for studying the inception period in plant species that do not tolerate temperatures sufficiently great to kill the bacteria was recently developed (21, 24). It had been shown in 1926 (168) that good tumor formation was obtained in tomato plants at temperatures up to 28°C., while no tumors were produced in this host at or above 30°C. Both host and bacteria developed well at or somewhat above 30°C. The underlying reason for this interesting finding remained obscure for more than twenty years. By applying the two-phase concept of tumor formation to this and similar systems, it has been shown (21, 24) that a temperature of 30°C. or more prevents the conversion of normal cells to tumor cells but does not interfere with multiplication of tumor cells once the cellular alteration has been accomplished. The process of cellular alteration could be brought to an abrupt and complete halt at any desired time by simply placing and holding inoculated plants at or above 30°C. The size and rate of growth of tumors that subsequently developed at 30°C. reflected the degree of cellular alteration that had occurred at a lower temperature (25°C.) up to the time the plants were placed and held at 30°C. With the use of this method a number of facts relating to the process of tumor inception were established. The inception period was accurately defined for two plant species (21, 24). There was a lag of about 34 hr. between inoculation of bacteria into a host and the first evidence that the cellular alteration had occurred. Tumors of the largest, most rapidly growing type were initiated only after the bacteria had acted for 60 to 72 hr. The host cells were found, for the most part, no longer susceptible to transformation 5 days or more after wounding despite the fact that many virulent bacteria were in contact with the cells after the fifth day. It is clear, therefore, that the inception period is of short duration and that it is during this period that temperature plays an important role. These

results have been confirmed with the use of this and other methods (48, 49, 183). To account for these findings Segretain (183) suggested that a heat-labile substance secreted by the cells during cicatrization may play a part in tumor formation

It was shown, further, (21) that the inability of the bacteria to accomplish the cellular alteration at or above 30°C. was not the result of physiological disturbances in the plant as a whole but was dependent upon environmental conditions that existed in the immediate vicinity of the point of inoculation. Wound healing occurred both at 25°C. and at 30°C.

The period necessary for the bacteria to alter normal cells to tumor cells could be reduced from 34 hr. to 10 hr. if the bacteria were permitted to establish themselves in a host for 24 hr. at 32°C. following inoculation (24). It should be recalled that the bacteria multiply well at and somewhat above 30°C. but are incapable of accomplishing the alteration. Periods of less than 10 hr. at 25°C. were inadequate, whereas progressively larger tumors resulted as the period of time was increased from 10 to 30 hr. at 25°C. following a 24-hr. incubation at 32°C. Since tumors initiated in 10 hr. at 25°C. following a 24-hr. incubation at 32°C. were essentially of the same size as were tumors that developed when a total incubation of 34 hr. at 25°C. was permitted, it was concluded that the first 24 hr. after inoculation represented either an incubation period necessary to permit the bacteria to establish themselves in the host or that it represented a period necessary to render the host cells susceptible to transformation. Subsequent work (29) showed the latter to be true.

In further experiments (24) plants were alternately subjected to temperatures of 25°C. and 32°C. following an initial incubation period of 24 hr. at 32°C. Five 6-hr. periods at 25°C. were alternated respectively with 1, 2, 4, or 6-hr. periods at 32°C. It was reported that when five 6-hr. periods at 25°C. were alternated with 1-hr. periods at 32°C., large tumors formed. As the intervals that the plants were held at 32°C. were increased in relation to the time they were held at 25°C., the size and rate of development of the resulting tumors decreased. When total exposures at 32°C. equaled those at 25°C., occasional small tumors or, in most instances, no tumors at all developed. On the basis of these results it was suggested that: (a) the TIP is cumulative as evidenced by the fact that, whereas a single 6-hr. exposure at 25°C. was inadequate to accomplish the alteration, a series of such exposures interrupted by short periods at 32°C. permitted the alteration to occur; (b) the TIP is inactivated at the higher temperature because if no inactivation had occurred at the higher temperature, the size of tumors resulting from the different exposures at 32°C. should have been much more equal because in each instance the same total of 30 hr. at 25°C. was given; (c) the TIP was inactivated or destroyed at 32°C. at about the same rate that it was produced at 25°C.

By careful control of temperature (25) it was found that measurable inactivation was limited to less than 2°C. Despite this narrow range reaction

rates were established. Since it was assumed in this study that inactivation was due to thermal destruction, the activation energy for this destruction was computed according to the Arrhenius equation. Very high values of the order of 80,000 cal./mole were obtained. Since reactions of this order of magnitude are characteristic of protein denaturation, it was suggested that either the TIP itself or something intimately associated with its inactivation may be a factor of complex structure. Needless to say, the correctness of this suggestion rests on the validity of the assumption that was made in this study.

Role of the wound in tumor inception.—It was recognized very early in the study of the crown gall disease that a wound is essential if primary tumors are to form. This notion has with few exceptions (185) been repeatedly supported by experimental evidence. Smith (187) speculated that a wound was required to permit entrance of bacteria into injured cells. De Ropp (56) considers the possibility that only those cells whose protoplasts are exposed as a result of a wound undergo transformation. Other workers have disagreed with this interpretation (166, 181).

It has been found in general that the ultimate size of the tumor is, within limits, proportional to the size of the initial wound (74, 80, 122, 166, 167). Hildebrand (80) suggested that the greater amount of wound sap present in large wounds was related to the massive size of tumors produced when such areas were inoculated with bacteria. The addition of plant sap to very small wounds appeared, moreover, to favor the development of somewhat larger tumors than would otherwise have been produced (74, 80). These studies suggested a possible role for wound sap in the genesis of crown gall tumors.

It is generally agreed (24, 49, 169) that plants become resistant to infection by the crown gall bacterium subsequent to the appearance of wound callus. Bacteria added without wounding to the surface of actively proliferating wound callus tissue were found to have no added effect (166, 168, 170). This has been interpreted to mean (49) that the callus tissue forms a mechanical barrier and thus closes the infection court to the bacteria. That interpretation, however, does not appear to acccount for findings described earlier in this discussion in which cells were resistant to alteration 4 to 5 days after wounding despite the fact that the bacteria were within the tissues in intimate contact with the host cells.

The etiological role of the wound healing process in tumor genesis has recently been established. These studies demonstrated (29) that the host cells must be conditioned by the stimulus of a wound before they can be altered to tumor cells. This effect was demonstrated quite simply by comparing the response of wounded tissue that had been permitted to heal for 48 hr. prior to inoculation with the response obtained when bacteria were introduced directly into previously unwounded tissue. In both instances the bacteria were allowed to act for only 24 hr. at 25°C. Large, rapidly growing tumors resulted when the bacteria acted for 24 hr. at 25°C. following a 48-hr. healing period, while no tumors at all were produced in that time when the bacteria were inoculated into previously unwounded tissue. Conditioning of the host

cells was shown to take place gradually reaching a maximum between the second and third days after a wound is made and declining again as wound healing progresses toward completion. This process was found, moreover, to take place independently of the bacteria at temperatures of both 25°C. and 32°C. although the cellular alteration was accomplished only at the lower temperature.

By comparing these findings with histological studies of the wounded area during the inception period, it was shown that it is just before or during the earliest stages of active wound healing that normal cells are altered to tumor cells of the most rapidly growing type. It is at this stage that the host cells show a high rate of metabolic activity. Conditioning, however, appears to be related to the wound healing reaction rather than with actively metabolizing cells generally. This is evidenced by the fact that, whereas cells that have been conditioned for 48 hr. prior to inoculation with bacteria can be altered to tumor cells of the rapidly growing type in 24 hr. at 25°C., unconditioned but actively metabolizing meristematic cells of an apical bud are not generally altered to tumor cells in that period.

As a result of a wound profound changes in the physiological behavior of the essentially resting cells in the region of the wound occur (11, 12). These modifications include changes in the permeability of the cell membranes, an increase in cyclosis in affected cells as well as respiratory modifications. Doubtless many other as yet uncharacterized changes accompany the activation of the resting cells. The specific role of such changes in the conditioning of cells for transformation in crown gall remains at present unknown.

Possible role of growth substance in tumor genesis.—The etiological role of the tumor-inducing principle elaborated by the bacteria and of conditioned host cells has now been clearly established in tumor genesis. Recently Klein & Link (96) have presented studies which they have interpreted to mean that a third factor, bacterial growth substance, is implicated as a "cocarcinogen" in the transformation process. Link, Wilcox, & Link (136) had earlier suggested that auxin was perhaps one entity in a complex of causal factors. Others workers (173) concluded that crown gall bacteria were pathogenic independently of auxin production. In 1942 Braun & Laskaris (18) discussed the two-phase concept of tumor formation and stated:

> The second phase consists in the stimulation of the changed cells to continued multiplication by a growth substance . . . the difference between attenuated and virulent strains may be in the relative amounts of growth substance produced by each either as a product of bacterial metabolism or by the host cells under the influence of these organisms.

In subsequent discussions (22) Braun has favored the latter view.

According to Klein & Link (96) the affected cells acquire as a result of the action of the TIP only the potentiality for autonomous growth but not the capacity for rapid duplication. Bacterial auxin is needed as a "cocarcinogen" to complete the transformation process and permit rapid multiplication of cells. In order to establish this concept experimentally it would, however,

appear necessary to show that growth substance-stimulated cells altered by an attenuated culture reached and retained indefinitely the high grade of neoplastic change found in cells altered by a virulent culture. This has not yet been done. These and similar results published earlier (96) have been interpreted differently. It has been suggested (22, 67) that crown gall tumor cells themselves acquire as the direct result of the action of the TIP the capacity for producing, in greater than regulatory amounts, a growth-promoting substance. Cells altered by an attenuated culture represent a lower grade of neoplastic change than do cells altered by a virulent strain. It is believed that by supplying an external source of growth substance to cells altered by an attenuated culture, those cells are being supplemented with biologically active materials that are elaborated in abundance by cells altered by a virulent culture. Additional studies will be needed to clarify this point.

The biochemistry of tumor inception.—Only those changes in biochemistry found to occur during the first five days after inoculation of a plant by wounding will be considered in this section. The papers dealing with this important phase of tumor formation are very limited in number and are for the most part of recent origin.

Link & Goddard (139) made a comparative time course study of the oxygen uptake of normal tomato hypocotyl and of the same organ inoculated with crown gall bacteria. It was reported that the rates of oxygen uptake in the infected tissues were, during the inception period, always greater than those of normal tissue controls when measured on a fresh weight basis. In contrast to these findings, Klein (94) reported a slight but significant decrease in oxygen uptake as well as a slight increase in fermentation when the inoculated tissues were compared with the controls two days after wounding. These differences were consistently found when data were plotted on a wet or dry weight, total phosphorous, or desoxyribonucleic acid (DNA) phosphorous basis. When plotted on a total nitrogen basis no such differences were noted, however.

In a continuation of these studies certain nitrogen and phosphorous compounds present in normal and potentially tumorous tomato tissues were compared. Two days after inoculation of plants with bacteria, protein and soluble nitrogen levels as well as total phosphorous had increased in the tumorous tissue above that found in the controls. Of particular interest was the increase found in DNA and protein phosphorous. The DNA peak was reached within 24 to 48 hr. after inoculation, followed by a drop of DNA level to that of the control within 5 days. The concentration of ribonucleic acid (RNA) in inoculated tissue followed that of the control tissue.

In subsequent studies (97) these findings were confirmed and extended to the broad bean. In this system the DNA peak was reached in 24 hr., maintained until 48 hr., and then returned to control level by 72 hr. after inoculation. It was shown that this peak was not due to increase in host cell numbers, bacterial nucleic acid content, or the amount of DNA in host cells as determined by cytochemical methods.

Additional studies with the tomato (98) showed that sterile wounding or

inoculation with avirulent bacteria did not result in a DNA peak between the second and third days after wounding. The inoculation of virulent bacteria into tomato plants held at 30°C., a temperature at which the cellular alteration does not occur, was followed within 24 hr. by a DNA peak which reached a level roughly half of that found in the same period by this organism at 25°C. At 30°C. the DNA peak dropped rapidly after 24 hr. to control levels.

The magnitude and shape of the DNA curve for tissues held at 30°C. following inoculation with virulent bacteria suggested that the DNA is either being inactivated or depolymerized at somewhat slower rates than it is being formed, or that is is being produced at reduced rates. Klein (98) appears to favor the former view.

Since the properties of the DNA corresponded well with the postulated biological and structural properties of the TIP and since the DNA reached its maximum concentration at a time when TIP is known to be active, Klein has suggested that this substance may, in fact, be identical with TIP.

Some critical experiments remain to be carried out, however, before this interesting suggestion can be accepted. It is known, for example, that the cells of the tomato plant (the host used by Klein) remain conditioned and are capable of being altered to tumor cells for a period of at least 2 weeks after wounding. Tomato cells respond differently in this respect than do cells of other hosts studied. It must be assumed on the basis of Klein's hypothesis that a given concentration (150 to 200 μg. DNA-phosphorus/g. dry wt.) of nonhost DNA-phosphorus must be reached before the cellular alteration will occur. An interesting question could therefore be posed. If, for example, tomato plants were inoculated with virulent bacteria and placed immediately at 32°C. for 3 days to prevent cellular alteration but to permit the development of the DNA peak and its subsequent decline to control levels, and if, then, after the third day the plants were placed at 25°C. to permit the cellular alteration to occur, would a second DNA peak develop as the host cells were altered to tumor cells? Klein's data suggest that it probably would not. If such were the case, it would indicate either that Klein's hypothesis, on the basis of the data presented, is wrong or that perhaps the TIP is produced by the bacteria very slowly and in very small amounts after the third day. If it is assumed that it takes much longer for bacteria to alter cells of tomato after the third day than before (and there is no evidence for this), then the effect of DNA on the cells may be cumulative over an extended period without at any time showing a sharp DNA peak. This possibility can also be tested experimentally.

THE DEVELOPMENT OF THE CROWN GALL TUMOR

The characteristic feature of the second phase of tumor formation is the continued unregulated proliferation of the altered host cells which ultimately results in a neoplastic overgrowth. The development of crown gall tumors in a host plant is apparently largely a function of the capacity of the host to nourish the tumor cells. A number of workers (117, 119, 168) have shown that tumor development is greater on a vigorously growing host than on a

poorly growing host. Flowering and fruiting were shown by Stapp & Bortels (204) to inhibit tumor formation appreciably in two host species studied. It was found, moreover, that inoculations of crown gall bacteria into apple trees that were making little or no growth did not induce the disease until the trees began to grow actively (171). Tumor development was shown by de Ropp (49) to be dependent on available carbohydrates. The growth of tumor cells could be almost completely suppressed by depriving isolated sunflower stem fragments of sugar for 5 days before inoculation with bacteria. This same worker showed that when crown galls are present on storage organs such as carrot or potato, the tumor possesses a remarkable ability to utilize the stored food materials (55). In contrast to earlier findings, Link *et al.* (141) recently concluded that, given suitable concentrations of essential nutrients, crown gall tumors grow at characteristic rates only slightly affected by the nutrition, vigor, or growth behavior of the host. These results indicate a very real capacity of crown gall tumors to mobilize nutrients from the host even when these factors are limiting in the host. Contrary to statements frequently found in the literature, crown gall development is not, within broad limits, influenced by temperature. It is the inception rather than the developmental period that is temperature-dependent.

The truly independent nature of crown gall tumor cells was first conclusively demonstrated by isolating fragments of bacteria-free tumor tissue from secondary (20, 223) and later from primary tumors (45, 66, 68, 85, 155, 224) and planting the sterile tissues on a suitable culture medium. These tumor fragments grew profusely and in a completely uncoordinated manner. Normal tissue fragments of the type from which the tumor tissue had originally been derived grew poorly on this medium and their growth was limited. It was clear, therefore, that a profound and permanent change had occurred in the physiology of the tumor cells. Following their alteration, the neoplastic cells possessed less exacting growth requirements than did normal tissues of the same kind. It was soon found, however, that this deficiency could be largely overcome in certain normal tissues by the addition to the basic culture medium of growth substances of the auxin type. When such growth substance-stimulated normal tissues were again removed to the auxin-free basic medium, however, cellular proliferation soon stopped. Although certain normal tissue was stimulated by the presence of auxin, tumor tissue was shown by de Ropp (46) and Hildebrandt & Riker (84) to be strongly inhibited by even relatively low concentrations of auxin. Low concentrations of auxin had suppressive effects on the oxygen uptake of tumor tissues (62, 142). This was found not to be reversed by the addition of organic acids to the system (62). The failure of even small amounts of auxin to stimulate growth of tumor tissues suggests that optimal amounts are already present in the tumor cells. De Ropp (47, 50, 51) demonstrated further that growth substances elaborated by tumor tissues greatly stimulated growth of normal tissues. These and other findings led in 1947 to the rather obvious suggestion (22, 67) that crown gall tumor cells themselves had acquired as a result of their transfor-

mation a capacity for producing in greater than regulatory amounts a growth-promoting substance. The continued and unregulated production of such a substance by the tumor cells was believed capable of accounting for the continued unregulated proliferation of those cells.

Numerous further attempts have been made in recent years to implicate auxin as the etiological agent responsible for the continued abnormal proliferation of the tumor cells. Bitancourt (7) reported the presence of indoleacetic acid-inactivating enzymes in normal tissue but not in tumor tissue. Henderson & Bonner (78), on the other hand, found no detectable indoleacetic acid-inactivating enzymes in either normal or tumorous sunflower tissue. They did, however, report the presence of an inhibitor in normal sunflower tisue which prevented the conversion of tryptophan to indoleacetic acid. This inhibitor, which was absent in tumor cells, while effective in the presence of low concentrations of tryptophan, was much less effective at high tryptophan levels. The studies of Bitancourt indicate then that the high auxin level found in crown gall tumor cells is the result of decreased auxin destruction. Those of Henderson and Bonner suggest, on the other hand, that this effect may result from uninhibited auxin synthesis. This phase of the problem requires re-examination.

Roberts (180) has recently presented evidence to show that when crystals of a naturally occurring but as yet chemically uncharacterized antiauxin were inserted into tomato plants prior to inoculation with bacteria, early growth of the tumors was somewhat inhibited and leaf epinasty, adventitious rooting, and cambial activity were greatly suppressed. The results reported with the use of maleic hydrazide, another antiauxin, have been conflicting, however. Waggoner & Dimond (222) found a significant reduction in the size of crown gall tumors on tomato and carrot following treatment of these hosts with maleic hydrazide. Kulescha (106) reported that fragments of *Scorzonera* crown gall tumor tissue were inhibited in the presence of a concentration of 10^{-7} of maleic hydrazide, while a concentration of 10^{-4} of this substance completely stopped their development. The auxin content of the tissues did not appear to be influenced by the treatment. Similar findings were reported by Manil & Straszewski (150). Klein & Klein (95), on the other hand, noted no such differences in tomato although the effects of maleic hydrazide had reduced the vegetative growth of treated plants by about 50 per cent. Nickell (165), working with a virus-induced tumor of *Rumex*, reported that 5 to 6 p.p.m. of this antiauxin incorporated in a culture medium was sufficient to reduce the growth of that tumor tissue by 50 per cent.

In 1935 Skoog (184) and later Gordon & Weber (72) found that auxin synthesis in higher plants is reversibly suppressed at low radiation doses and irreversibly inhibited at higher radiation dose levels. In view of these findings it is interesting to note that a number of workers have reported inhibitory effects of radiations of various kinds on the development of crown gall tumors. As early as 1922 Levin & Levine (115) found that radium emanation tubes inserted into crown gall tissue suppressed the development of the

tumor. Later studies by Levine (120) showed that capillary tubes containing 0.3 to 0.6 mc. of radium when inserted into stems of *Geranium* 1 to 2 days after inoculation prevented tumor formation. Similar results were obtained by Rivera (179) and by Arnaudi & Venturelli (1). Rivera (178) indicated, further, that small doses of x-ray exerted at first a stimulatory and later a lethal effect on crown gall tumors of *Ricinus* and *Pelargonium*. With larger doses tumor development was arrested and regression of the overgrowth soon followed. More recently Waggoner & Dimond (221) found that crown gall formation on several host species was suppressed when the hosts were exposed to 30,000 r of gamma radiation. X-radiation equal to or greater than 4,000 r suppressed tumor formation in the tomato. The appearance of the overgrowths was delayed a few days by 4,000 to 5,000 r and for 3 weeks or more by doses of 6,000 r or more. Crown gall bacteria exposed to 10,000 r were still found to be pathogenic. Since the relation between radiation dose and delay in tumor formation was found to be similar to that reported by Skoog and Gordon and Weber for the relation between radiation dose and auxin production, it was suggested that the radiation-caused delay in tumor formation may be due to a suppression by radiation of auxin production by the tumor cells. This interesting hypothesis has not yet been tested experimentally. These data together with those reported earlier in this discussion suggest that auxin synthesized by the tumor cells may serve as an etiological agent in the proliferation of those cells. Whether the characteristic behavior of crown gall tumor cells can be explained solely or, even in large part, on the basis of high and unregulated auxin levels in those cells remains to be determined, however.

The development of methods for growing bacteria-free crown gall tumor tissues on a chemically defined culture medium led to numerous physiological and biochemical studies with the use of these tissues. The optimum physical and chemical environment (81, 83, 85) for the growth of several tumor species has been defined. Dextrose, levulose, and sucrose were found most suitable as sources of energy. Some tumor species grew quite well while others failed to grow when mannose, galactose, lactose, arabinose, and starch were used in the medium as the sole source of carbon. All tissues grew poorly on pentose sugars, alcohols, and organic acids. Of the nitrogen sources tested, nitrate and urea were found most suitable. Nitrite nitrogen and 11 amino acids permitted no growth (176). When nitrate was present in the culture medium, growth was sharply inhibited by nitrite, asparagine, and all amino acids tested when the latter were used in concentrations of $0.001 M$. It was found, however, that growth resulted with higher ($0.064 M$) concentrations of alanine, aspartic acid, and glutamic acid. Parallel studies have been carried out on virus-induced tumors of sorrel by Nickell, Greenfield & Burkholder (162, 163, 164). Most notable in these studies is the very high requirement of the virus tumor tissue for phosphorus and thiamine and the secretion of α-amylase by these tissues (16).

Stimulatory substances.—Extracts of crown gall tumors of tomato, marigold, and of yeast stimulated somewhat the growth of the sunflower tumor

tissues in culture when used in low but not high concentrations (82). A slight increase in fresh weight of tumor tissue was observed in the presence of very low concentrations of cysteine hydrochloride as well as certain other of nine growth-regulating substances tested (84). Morel (155) found that tobacco tumor tissue but not other crown gall tumor tissue studied was stimulated by low concentrations of indoleacetic acid.

Although Henderson, Durrel & Bonner (79) found no stimulatory effect of coconut milk on the growth of sunflower tumor tissue, Duhamet (59, 60, 61) reported pronounced stimulation of a number of other tumor species. Tumors of *Scorzonera*, for example, more than doubled their growth rate in the presence of 50 per cent coconut milk. Kovoor (101) indicates that 5 per cent filtered white grapejuice when added to the culture medium lends a strong impetus to the proliferation of this tumor tissue. Work recently reported by Bouriquet (15) is of interest in this connection. Bouriquet found that crown gall tumor tissue of *Scorzonera* could be greatly stimulated by mercaptoethylamine as well as by mercaptothiazoline. The former is of particular interest because of its relationship to coenzyme A. The two compounds in question were found to exert no comparable stimulatory effect on normal carrot root tissue, the vascular parenchyma of the Jerusalem artichoke, or on wheat or radish seedlings. The fact that Jersualem artichoke tissue, which is incapable of proliferating in the absence of indoleacetic acid, was not stimulated by these compounds suggest, that the latter do not possess auxinic activity.

Inhibitory substances.—Many substances have been found to be inhibitory to the growth of crown gall tumor cells. Among those already mentioned are the auxins. Colchicine (37, 202) was found capable of arresting proliferation of crown gall tumor cells as were certain dyes such as malachite green and analine blue which combine with the nucleic acids (58). Of the antibiotics tested, penicillin and, more particularly, streptomycin were found effective in inhibiting growth of the tumor cells (34, 35, 76). De Ropp (53) showed that penicillin G was effective at a concentration of 500 μg./ml., while streptomycin inhibited growth of these tissues at a concentration of 50 μg./ml.

Some of the most active inhibitors of tumor cell proliferation were found by de Ropp (52, 54, 57) to be analogues of pteroylglutamic acid. The analogues containing a methyl as well as a 4-amino group partially inhibited the growth of tumor tissue at a concentration as low at 1 μg./l. Ten times that concentration was required for other effective analogues. The growth of certain normal tissue was, however, inhibited even more strongly than was the tumor tissue by these compounds. The inhibition was not, moreover, reversible with the use of pteroylglutamic acid. Folinic acid was not tested in these experiments. Cytological studies showed that these compounds exerted their inhibitory effects by preventing cell multiplication rather than cell enlargement. Cortisone and several nitrogen mustards as well as guanazolo were also found to be inhibitory to the growth of crown gall cells *in vitro*.

In an interesting study published about 20 years ago, Gosset, Magrou &

Tchakirian (73) showed that when germanium dioxide was introduced into the general circulation of a test plant bearing crown gall tumors, subsequent necroses of the tumors occurred in many instances without harmful effects to the host plant. More recently, Magrou & Manigault (147) reported that permanent magnetic fields inhibit tumor development by suppressing cell proliferation.

THE BIOCHEMISTRY OF CROWN GALL TUMOR DEVELOPMENT

Biochemical studies dealing with constituents and activities of crown gall tumor and normal tissue have, with few exceptions, compared actively proliferating tumor cells with essentially resting cells of a comparable organ of the same plant species. Under such experimental conditions one would indeed expect to find qualitative differences betweeen tumor and normal tissue. The possible etiological significance of such differences in tumor development is extremely difficult to evaluate, however. In any study of this kind it would appear desirable to cultivate *in vitro* bacteria-free crown gall tissue and normal tissue obtained from exactly the same host species on the same chemically defined medium and under precisely the same conditions. In this way qualitative or quantitative differences found in the constituents and activities of these sterile tissues could be correlated with reasonable certainty with the tumor character of one of the tissues. Only a few of the more recent studies have followed that procedure.

Carbohydrate metabolism.—The sugar content of tumors of the sugar beet and tomato was found by a number of workers (157, 158, 205, 209) to be considerably less than that found in healthy root or stem tissue. The starch concentration, on the other hand, was highest in the tumor tissue of these hosts. Pectin and cellulose were present in slightly higher amounts in beet tumors than in beet roots and in slightly lower concentrations in tomato tumors than in normal tomato stem tissue.

Neish & Hibbert (159) attempted to account quantitatively for the total carbohydrate metabolized during a 3 hr. period of respiration in beet tumor and normal beet root slices. In the tumor 25 per cent of the sugar metabolized was accounted for by the CO_2 evolved, while the fate of the remainder was unknown. In normal beet root tissue, on the other hand, the CO_2 evolved accounted for 19 per cent of the sugar metabolized, while the combined oxalic, malic, and citric acids found represented a further 52 per cent, the remaining 29 per cent being unaccounted for. Under anaerobic conditions an alcoholic fermentation occurred in tumor slices which accounted for about 70 per cent of the sugar metabolized. No detectable lactic acid producion was observed although a small increase in keto acids was found. The metabolism of normal tissue slices under these conditions was quite different. Both alcoholic fermentation (19 per cent) and lactic acid formation (24 per cent) occurred. These account for 45 per cent of the sugar metabolized under anaerobic conditions. Normal tissue but not tumor tissue showed a decrease in the rate of carbohydrate catabolism when air was admitted to the fermenting

tissue. Thus, the two tissues can be differentiated by the existence of a lactic acid fermentation and a Meyerh of effect in the normal but not in the tumor tissue. A pronounced Pasteur effect was found in both.

Nitrogen changes.—It is generally agreed that marked differences exist in the nitrogenous constituents of normal and tumor tissue (157, 158, 205, 209). Neish & Hibbert (160) found, for example, that, while about the same amount of nonprotein nitrogen exists in beet tumor and normal tissue, the tumors have a protein content of about three times that found in the normal tissue. This increase in protein content was accompanied by a corresponding decrease in sugar content and it was suggested that the high level of sugar utilization by the tumor tissue indicates that the carbohydrates are being used not only for cell wall formation but for the specific synthesis of protein. Similar findings have been reported for this and other hosts. Water-soluble protein was found by Neish & Hibbert (160) to be six times that present in the normal tissue. The tumors, moreover, maintained 64 per cent of their Kjeldahl nitrogen in the form of protein as compared with only 39 per cent for normal tissue, thus reflecting a greater tendency of the tumors to synthesize protein.

The effects of the addition of ammonium sulfate on the aerobic metabolism of the tissue slices of the two types of tissue was further studied. Fixation of some of the ammonium ion as glutamine and asparagine was found to occur in both tissues while protein synthesis occurred in tumor but only to a very slight extent in normal tissues. Under anaerobic conditions, on the other hand, protein synthesis did not occur in either tissue but the ammonium ion was found to be fixed as glutamine and asparagine.

Lee (111) has recently made a comparative study of certain nitrogenous constituents present in crown gall, habituated, and normal tissue of the European grape. No qualitative differences in the amino acid composition of these tissues was found. The crown gall tissue had the highest concentration of total nitrogen and soluble nitrogen, while normal tissue had the smallest amount of these constituents. Habituated tissue had intermediate levels.

Klein (94) and, earlier, Klein & Keyssner (91) reported that the major increase in protein nitrogen as well as soluble nitrogen was found in tumor tissue relatively late in the development of the tumor. Since soluble nitrogen increased at this late stage at a considerably slower rate than did protein nitrogen, it was suggested (94) that the synthesis of protein does not occur entirely at the expense of amino acids and amides. It was suggested, further (94, 141), that the crown gall tumor possesses a pronounced capacity to mobilize nitrogen from the host even though the host itself may be greatly deficient in that element.

What appears to be a most significant finding was recently reported by Camus, Wildman & Bonner (41), and discussed by Gautheret (70). The results of this study suggest that crown gall tumor tissue contains a new high molecular weight protein that constitutes about 20 per cent of the total protein. This new protein was found to be absent in normal and habituated

tissue of the same kind. Needless to say, the etiological implications of this finding may be very great. A comprehensive report of this work has not yet appeared.

Phosphorus changes.—Total phosphorus of tumor tissue has been reported by a number of workers (94, 111, 148, 158, 205) to be considerably higher than that found in normal tissue. Phosphorus supplied as P^{32} was found by Tsao & Whaley (214) to be accumulated more extensively in tumor tissue than in normal tissue of *Bryophyllum*. The concentration of total phosphorus was somewhat greater in tumor tissue than in stem apices and much greater than in mature stem tissues. In a time course study Klein (94) reported increases in phosphorus-containing compounds in tumor tissue as early as 5 days after inoculation. The increased concentration was found to be due to increased amounts of orthophosphate and acid-soluble organic phosphates. Within 14 days after inoculation the total phosphorus was twice that of the control and over half of this increase was due to an increase in the acid-soluble organic fraction. During later stages of tumor development the relative concentration of the acid-soluble organic fraction decreased until at the time of maturation of the tumor this fraction formed about the same proportion of the total phosphorus as was present in the control.

Respiration.—Neish & Hibbert (159) reported the respiratory quotient of normal and tumor tissue of the sugar beet to be 0.64 and 0.92, respectively. The low R_Q of the normal tissue was explained by the formation of citric, malic, and oxalic acids. Calculations showed that the amount of these three acids accounted for about 75 per cent of the oxygen consumed in excess of that required for an R_Q of unity. Since such acid formation does not occur in the tumor tissue, the experiments suggest a marked difference in the metabolism of the two types of tissue. The addition of certain organic acids were found by Eberts, Burris, & Riker (62) to increase significantly rates of oxygen uptake in washed slices of tomato tumor tissue and normal tissue slices. In no case did the organic acids reverse an inhibition induced by indole-3-acetic acid. These workers reported further that the Q_{O_2} (N) (microliters oxygen consumed per mg. tissue nitrogen per hr.) of exhaustively washed slices of old tomato tumor tissue was lower than that of healthy stem slices similarly treated. Klein (94) obtained similar results only when the tumor tissues were quite old. In 1945 White (225) studied rather extensively the respiratory behavior of a number of bacteria-free tumor tissues of the sunflower. The bacteria-free tumor tissues were found to have a somewhat lower Q_{O_2} than did the normal tissue used. On the basis of these findings, White concluded that the various tumors studied did not show significant qualitative changes in respiration but did show a definite lowering of respiratory levels. It was pointed out, however, that these conclusions, based on dry weight, may be misleading. On the basis of their studies, Berthelot & Amoureux (4) suggested that aerobic fermentation occurs in beet tumors. Link & Goddard (139) compared, on a wet weight basis, tumor and normal tissue of the tomato and

concluded that the rates of oxygen uptake of tumor tissue slices were at all stages in the development of the tumor greater than those found in the normal controls. Klein's (94) findings are in essential agreement with those of Link and Goddard except during the inception period of tumor formation at which period Klein found a somewhat lower oxygen uptake in the tumor tissue. Link & Goddard (139) reported that the rate of oxygen uptake of tumor tissue derived from nitrogen-deficient plants was at the same level as that found in tumors on plants supplied with adequate nitrogen. This was true despite the fact that the tumors developing on nitrogen-deficient plants contained less than half the total nitrogen found in tumors grown on plants receiving an adequate supply of nitrogen. It is clear that differences in respiration of tumor and normal tissue must be based on some constant cellular character if real differences are to be found. Total nitrogen does not appear to be such a character since nitrogen has been shown to accumulate in older tumors. Klein has suggested that desoxyribonucleic acid might be the base of choice in plants that do not show polyploidy.

Enzymology.—A number of investigators have shown that catalase, peroxidase, and oxidase (tyrosinase) activity was higher in tumor tissue obtained from a number of plant species than in comparable normal tissue (77, 92, 93, 157, 169, 205). The increased catalase activity found by Klein & Ziese (92) was shown to be a function of the tumor tissue rather than the pathogen since the crown gall bacterium itself possessed only a very slight catalase activity. Nagy, Riker & Peterson (157) reported the presence of an active tyrosinase in tumor tissue but not in normal tissue of tomato. Levi, Michaelis & Hibbert (112) found tyrosinase and peroxidase to be far more active in tumor tissue of the beet than in normal tissue. Ascorbic acid oxidase, on the other hand, was less active in tumor tissue. It was concluded that the increase in concentration of ascorbic acid in the tumor tissue was the result of a decreased activity of its oxidase. Studies based on cyanide-sensitive respiration indicated that the heavy metal enzyme systems mediate most if not all of the oxygen uptake of both normal and tumor beet tissue. Link & Klein (140) reported that while Fe, Cu, and "residual" metallo-protein enzymes are each responsible for about one-third of the oxygen uptake of normal tomato stem tissues, the Cu (tyrosinase) systems account for three-fourths of the oxygen uptake in old crown gall tumors.

Secondary Tumors

In addition to the primary tumor there may be produced in certain host species such as the sunflower (17) and the Paris daisy (187, 188) secondary tumors that arise at points distant from the seat of the primary inoculation. These secondary overgrowths were first described by Smith (187, 188) and were believed by him to be comparable to certain types of metastases found in malignant tumors of animals and man. In Smith's opinion secondary tumors were always connected with the primary tumor by means of a tumor strand which grew in root-like fashion along the path of least resistance in the host.

Later, however, Smith (199) modified somewhat this concept of root-like invasion of normal tissue by the tumor strand and stated that, in some instances at least, the strand develops by appositional growth. Subsequent work has made both interpretations appear extremely unlikely. Another explanation for the mechanism of secondary tumor and tumor-strand development was advanced independently by Riker (167) and by Robinson & Walkden (181). It was contended by Riker that such secondary structures are never formed except when inoculations are made into the tips of plants having a number of internodes condensed in an apical bud. When inoculations are made just below the bud of such plants, the injured cells release liquid which forms a continuous channel and serves to distribute the bacteria along a number of nodes and internodes in the condensed bud. As the bud develops and the internodes elongate, bacteria are distributed for some distance along the stem. It was believed that tumor strands and secondary tumors were formed by the division of host cells along the path where the bacteria were distributed at the time of inoculation. That this concept was not entirely correct was demonstrated some years later by Braun (17). It was shown that both secondary tumors and tumor strands could be obtained when bacterial inoculations were made into fully elongated internodes at a distance of 6 in. from the apical bud in sunflower plants. Of special interest was the finding that many of the secondary tumors and tumor strands were sterile. This finding was in accord with earlier observations made by Smith (188). On the basis of essentially two lines of evidence, (a) secondary tumors and tumor strands may be produced in some instances by an attenuated culture that does not initiate the production of primary tumors, and, more particularly, (b) on the basis of histological findings dealing with the origin and development of these secondary tumefactions, it was suggested by Braun (17) that perhaps the bacteria remain confined to the xylem vessels and that under their influence substances are formed that diffuse laterally and bring about cellular disturbances in the vicinity of the vessels. This interpretation was recently severely criticized by Riker, Spoerl & Gutsche (175). They stated that "The bacteria have been present in vessels without causing galls until they were released into the surrounding tissues." To illustrate this point these workers cite experiments carried out by Riker (166) on tomato plants. Since secondary tumors and tumor strands of the type found in the sunflower have never been reported to occur in tomato, it seems rather surprising to find that conclusions should have been drawn on the basis of comparisons of the response to bacterial inoculation of these two very differently reacting host species. It was stated, further, "That bacteria have been in contact with several kinds of uninjured cells for long periods without causing galls." In making this point the critics had not considered the need for conditioned host cells in the transformation process. As sunflower plants are inoculated by needle puncture, juices from the ruptured cells are carried by capillarity in xylem vessels that have been broken as a result of the wound. Such plant juices are known to contain conditioning substances and, should such substances diffuse lat-

erally from the vessels, they would serve to prepare the cells for transformation. Histological findings have shown, moreover, that it is the cells in the immediate vicinity of such vessels that are altered to tumor cells. It was suggested further that perhaps the plants used by Braun were short-day, high-temperature plants. According to Riker and collaborators the air spaces of such plants frequently become flooded, thus furnishing a continuous channel of liquid from the primary tumor to the apical bud through which the bacteria could move. Since secondary tumors have been found to develop equally well in sunflower plants at all times of the year and under a considerable range of temperature, this argument does not appear sound. Histological findings, moreover, make the explanation quite untenable.

It appears that Riker has based his arguments essentially on the unproved assumption that tumefaction can occur only in the immediate presence of the bacteria. In those many instances in which the secondary tumors have been found to be sterile, he believes that the organisms were originally present but had died out. The actual observations are equally consonant with the alternative interpretation that the bacteria present in the tissues of certain secondary tumors were not responsible for the development of those growths. Bacteria which are known to be present in xylem vessels of inoculated sunflower plants may well have entered the region of the proliferating cells as secondary invaders from xylem vessels that had been ruptured as a result of stresses accompanying growth of the secondary tumor. Although the mechanism by which secondary tumors are initiated in the intact plant has not yet been clarified, de Ropp's (47, 50, 51) studies relating to the *in vitro* production of these structures are enlightening. This worker has shown that it is possible to obtain secondary tumors in normal tissue by grafting bacteria-free crown gall tumor tissue of the sunflower on normal sunflower stocks. These secondary growths show a histological structure which is strikingly similar to that found by Braun (17) to be present in bacteria-free nodular secondary tumors that develop in sunflower plants following inoculation with virulent bacteria. The secondary tumors found by de Ropp, like those studied earlier by White & Braun (223), are capable of profuse growth on a culture medium that does not support the continued growth of normal tissue. Similar results were recently obtained by McEwen (151). Work carried out by Camus & Gautheret (40) indicated that bacteria-free crown gall tissue of *Helianthus tuberosus* will also, when grafted to healthy tissue of this plant *in vitro*, cause the production of secondary outgrowths. De Ropp (56) has suggested that a principle exists in the tumor tissue itself which is capable of changing healthy cells to tumor cells. Whether this is the same principle originally elaborated by the bacteria, or a new principle developed in the tumor tissure as a result of bacterial action, is a matter for future studies to determine.

Bacterial Virulence and Tumor Morphology

It is not the purpose of the present paper to present a detailed description of the physiology of the crown gall bacterium. It will suffice to state that the

species *Agrobacterium tumefaciens* is composed of a heterogeneous group of strains that may differ markedly from one another in their serological, physiological, and pathogenic properties. Of interest, however, are some of the findings relating to the virulence or disease-producing capacity of these organisms. Some strains of the crown gall bacterium have a very wide host range while others are limited in their disease-inducing ability to single plant species. Isolates within individual strains may also possess very different capacities for initiating tumors. Some may be highly virulent, other attenuated in varying degrees, while still others may be completely avirulent.

In 1937 Longley *et al.* (145) reported the very interesting finding that the virulence factor in the crown gall bacterium was lost as a result of 15 to 20 successive passages of this organism in a glycine-containing medium. This work has been repeatedly confirmed and extended to include certain other amino acids as attenuating agents (215, 216, 217). The permanence of attenuation depended on the length of time that the cultures were kept on the attenuating medium. If such cultures were removed from the glycine-containing medium as soon as they had become avirulent for tomato and placed on a yeast-infusion medium, virulence was gradually regained. However, if cultures that had lost their virulence for tomato were carried through ten additional passages on a glycine-containing medium, their disease-producing ability could no longer be restored. Recently Stapp (207) has shown that as a result of fifteen passages of the *Dahlia* Ra strain in an α-alanine-containing medium, this strain had lost completely its tumor-inducing ability for tomato and *Pelargonium* but had at the same time retained almost undiminished its original virulence for *Datura*. By serial passage in a leucine-containing medium on the other hand, strain *Chrys. frut.* IIb lost its disease-producing ability for *Pelargonium* but retained a high degree of virulence for tomato and *Datura*. These findings suggest the interesting possibility that perhaps a number of tumor-inducing principles exist in a bacterial cell, each of which possesses a certain host specificity and which may be lost independently by the bacterial cells as a result of passage in media containing the attenuating amino acids.

Klein & Klein (90) in an extension of work reported earlier by Coleman & Reid (42) suggest that disease-producing ability can be transmitted from virulent to avirulent strains of crown gall bacterium as well as to related species. According to these workers the biologically active material appears to be a polymerized desoxyribonucleic acid.

Different strains of the crown gall bacterium may initiate the production of morphologically very distinct overgrowths when inoculated into the same host species. Stapp & Pfeil (205), for example, have pictured tumors incited by the *Chrys. frut.* IIb and *Dahlia* Ra strains on sugar beet plants. The tumors induced by the *Chrysanthemum* strain possess a highly convoluted surface, while the *Dahlia* strain produces tumors that have a smooth external contour. More striking are results obtained with the use of the T[37] walnut and B[2] strains on *Kalanchoe* (23). When these strains are inoculated close to the tip of this host, the T[37] strain initiates the production of

complex tumors or teratomata which are composed of a chaotic assortment of highly abnormal tissues and organs. The B^2 strain, on the other hand, gives rise to typical unorganized crown gall tumors in this host. Recent studies (28, 32) have shown, moreover, that even the same strain (T^{37}) of bacteria can itself induce the formation of morphologically distinct tumors on the same host. Complex tumors have, in the past been described many times on a number of different host species (23, 38, 107, 113, 114, 116, 118, 146, 193, 196, 198). The cells of these hosts were in all instances characterized by the fact that they possessed well developed regenerative capacities. There has been disagreement, however, as to whether the morphologically distorted but organized structures that develop from complex tumors are composed of normal tissue that has been carried along by the expanding tumor (196, 198) or whether the tumor cells themselves possessed the capacity to organize these structures (23, 113, 114, 116). Because of the lack of suitable methods this problem has been difficult to resolve. The development of tissue culture methods, however, provided a new approach with which to study this question. White's basic culture medium supports the profuse growth of crown gall tumor tissue but does not permit the continued proliferation of normal tissue. Hence, it could be made to serve as a differential medium. Bacteria-free cells isolated from morphologically organized but abnormal structures that developed from tobacco teratomata grew profusely on White's basic medium, as did typical unorganized crown gall tumors. The teratoma-derived cells differed from the commonly found crown gall tumor cells, however, in that they retained indefinitely in culture a highly developed capacity for organization. The surfaces of such cultures were covered with small distorted leaves and buds. The growth characteristics of this tissue in culture contrasted sharply with results that had been obtained earlier (27, 28) with the use of crown gall tissue isolated from typical unorganized tumors. Such tissue never showed the slightest tendency to organize and its capacity for differentiation was very limited. There appeared, therefore, to be similarities as well as basic differences between cells isolated from the two morphologically distinct crown gall tumors. In order to examine this question further a study was undertaken (32) to investigate the interrelationship of bacterial and host factors concerned in determining tumor morphology in crown gall. It was found in these studies that, when a tobacco plant was cut through an internode at about the middle of the plant and both freshly cut surfaces were inoculated with the moderately virulent T^{37} strain, typical unorganized crown gall tumors developed at the basal end of the upper cutting. The other inoculated surface that had become the tip of the lower half of the plant gave rise to complex overgrowths. The cells of the two cut surfaces prior to the time of their separation possessed the same potentialities since they were adjoining cells of the same stem. Immediately after the cut was made, however, the cells below the point at which the stem had been severed became apical cells of the basal portion of the original plant, while the cells above the cut became basal cells of the upper cutting. Bacteria-free tissue fragments isolated either from teratomas initiated at the cut stem tips or from typical unorgan-

ized tumors that developed at the basal ends of tobacco cuttings were similar in growth pattern when cultivated *in vitro*. Cells isolated from either type grew profusely in culture and retained indefinitely a capacity to organize small abnormal leaves and buds. Bacteria-free fragments of this type, when grafted to the tips of healthy tobacco plants, developed into complex tumors. Similar fragments implanted at cambial level into internodes of tobacco plants containing functional apical buds developed into typical crown gall tumors of the unorganized type. As a result of this study it was concluded that the ability of pluripotent tobacco tumor cells to organize is determined largely by the position that they occupy in the plant axis.

Tobacco tumor cells altered by the highly virulent B^6 strain instead of the moderately virulent T^{37} stain of the crown gall bacterium lost entirely both *in vitro* and *in situ* their capacity to organize structures. When bacteria-free fragments of such tumor tissues were grafted either at the cut stem tip or into internodes of a tobacco plant containing an apical bud, typical unorganized crown gall tumors resulted. It appears, therefore, that, although the tumor-inducing principle associated with the moderately virulent T^{37} strain is incapable of destroying the factors concerned with differentiation and oganization of pluripotent tobacco cells, the principle associated with the highly virulent B^6 strain completely overwhelms those factors. The difference in response of tobacco cells to the action of these two strains may be concerned with dosage of TIP at the time of cellular transformation. These results might also be accounted for by assuming that the TIP elaborated by the T^{37} strain is not identical with that produced by the B^6 strain.

Cells of such plant species as the sunflower, which do not possess the regenerative competencies of pluripotent tobacco cells, are irreversibly altered to tumor cells of the type incapable of organization both by the moderately virulent T^{37} strain and the highly virulent B^6 strain. Thus, although the TIP elaborated by the T^{37} strain is incapable of cancelling the organizational capacity of pluripotent tobacco cells, this principle does initiate the development of undifferentiated and unorganized tumors when plant cells possessing a low competency for regeneration are altered by it. It appears, therefore, that essentially three factors, (*a*) the strain of the crown gall bacterium used to render the cells neoplastic, (*b*) the relative position that the tumor occupies in the host, and (*c*) the inherent potentialities of the host cells for regeneration, may all be concerned in determining the morphological structure of the resulting overgrowth.

RECOVERY OF CROWN GALL TUMOR CELLS

Crown gall tumor cells of the commonly found type possess a high degree of neoplastic change. This is evidenced by their rapid and uncoordinated powers of proliferation, their very limited powers for differentiation, and their complete lack of a capacity to organize. Since such tumor cells, isolated from a number of taxonomically widely separated plant species, have not, during the more than five years that have elapsed since their isolation, shown the slightest tendency to become less autonomous, they have generally been

regarded as permanently altered cells and therefore unsuited for investigations on recovery. The recent finding (28, 32), however, that when pluripotent cells found in certain plant species are altered to tumor cells, those cells retain, despite their alteration, highly developed regenerative capacities, provided a necessary experimental tool for studies on recovery of crown gall tumor cells. Tumor shoots derived from tumor buds that developed from teratomata were forced into very rapid growth by a series of graftings to healthy plants. When these tumor cells were forced in this manner they gradually recovered and became normal in every respect (27, 28).

The organizational ability of these teratoma-derived tissues is an expression of the inherent potentialities of the pluripotent tumor cells and it does not in itself appear to affect directly the recovery of those cells. It is imperative, although this has not always been done in the past, to make a clear distinction between organization and recovery. Recovery of the affected cell appears to take place only when the cells of a tumor bud are forced to divide very rapidly.

The fact that it is possible to cause a reversion of tumor cells to completely normal cells as a result of unusually rapid growth suggests that the tumor cells contain within themselves all of the cellular factors, both genetic and nongenetic, that were present in the normal cell from which the tumor cell was originally derived. In addition, however, the tumor cell seems to have acquired as a result of bacterial action an additional factor which appears to be subject to the effects of dilution and is therefore lost in very rapidly dividing cells. Whether this factor is a virus or viral-like agent as suggested by the work of de Ropp (47) and Camus, Wildman & Bonner (41), or a new self-perpetuating combination of bacterial and host factors as hypothesized by Braun (33), or whether in fact, some entirely different mechanism is involved remains to be determined.

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TROPISMS AND NASTIC MOVEMENTS[1]

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Introduction

Four years have passed since the last report on plant tropisms appeared in the *Annual Review of Plant Physiology* [Schrank (32)]. The progress made during this period is relatively modest, particularly if compared with the very remarkable recent developments in the chemistry and physiology of the growth substances so closely related to our topic. As far as the present reviewer is aware, only the gradual elucidation of the photolytic inactivation of indole-3-acetic acid (IAA) initiated by Galston (12) seems to have had immediate influence on tropistic research by opening a promising new road to a better understanding of the mechanism of light-influenced growth reactions. In contrast, the analysis of the primary effect of gravitation in the geotropic reaction chain has hardly advanced beyond its descriptive stage, and, moreover, some originally hopeful approaches to a more rational concept of the situation had to be abandoned. The crux of the matter evidently lies in the fact that any direct relation between gravitation and chemical effect is still much less understood than the very obvious chemical aspects of light. This contrasting situation will show itself in many papers to be discussed in this article.

A comparison of the main trends in the past with those prevailing in the more recent work on active plant movements reveals one particularly remarkable change in the basic concept. While the intimate dependence of the mechanism of motion on the interaction of auxins is actually now stressed more than previously by admitting an auxin linkage even in the turgor movements of pulvini, the question of how the necessary differences in auxin concentration are brought about has lately been approached with much more caution. The assumption of a lateral translocation seems to have receded somewhat in favor of the view that the stimulus, by direct or indirect action, causes an asymmetric destruction or formation of growth substance.

In the previous review only tropisms *sensu stricto* were discussed. This time a somewhat larger field will be covered by including also some recent work on nastic movements.

Shortly before the present article was completed, a review on "Wachstum und Bewegung" appeared in the recent volume of *"Fortschritte der Botanik"* [Guttenberg (17)]. Thus, some of the earlier papers of the period under survey are discussed in both reviews; this may be no disadvantage, however, because it will help the reader to obtain a more objective picture of the cur-

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[1] The following abbreviations will be used: IAA, indole-3-acetic acid; Rfl, riboflavin; TIBA, triiodobenzoic acid; mcs., meter-candle seconds.

rent trends. The present paper carries the discussion two years further than Guttenberg's report, covering the period from 1950 to August, 1953.

PHOTOTROPISM

Galston's demonstration of the strong sensitizing effect exerted by ribo-flavin (Rfl) upon the photolysis of IAA had left still unanswered the important question whether, in natural systems, carotenoids can act as alternative catalyzers. Since accumulating evidence indicates that the main growth substance of the higher plant is IAA and its derivatives rather than the elusive auxin-*a*, Kögl's original conception of a carotene-sensitized transformation of active auxin-*a*-lactone into the inactive lumi-*a*-auxone has lost much of its material basis. The problem left, then, was whether carotenoids, also, could act as photocatalyzers of IAA. *In vitro* experiments to this effect were carried out by Reinert (30, 31). Using aqueous mixtures of IAA with colloidal solutions of β-carotene (prepared according to Kögl's prescription), he failed to discover any photolytic destruction of IAA in visible light. The same negative result was also obtained with true solutions of both substances in alcohol. Not satisfied with this, Reinert next examined the possible effect, in a different direction, which could be produced by the presence of carotene in the system. First, he showed that neutral growth substance (indoleacetaldehyde), which when illuminated loses its enzymatic transformability into IAA, retains this faculty if carotenoids are present during the irradiation. More impressive still is the fact that the addition of carotene to the reaction system IAA+Rfl almost completely inhibits the photocatalytic action of Rfl. This protective effect seems to be chiefly due to the similarity of the absorption spectra of both yellow pigments which, in the critical region between 400 and 500 mμ, largely overlap. Accordingly, the added carotene must act as an "internal" light filter, screening off the most effective wave lengths from the Rfl.

In discussing the implications of his findings for the phototropic conditions in the *Avena* coleoptile, Reinert stresses the fact that in this rather transparent organ carotenes are localized in the tip region only, while Rfl seems to be evenly distributed over the whole length. If this situation is duly considered, several hitherto contradictory phenomena can be satisfactorily correlated with one another: (a) the action spectrum for the phototropic response shows a double peak in the region between 400 and 500 mμ, thus corresponding to the absorption spectrum of a carotenoid rather than of Rfl; (b) the approximate coincidence of the region of maximal phototropic sensitivity with that of the highest carotene content; (c) the ability of decapitated coleoptiles to carry out almost normal light-growth reactions while their phototropic response is very poor.

By assuming that the light-growth reaction as well as the phototropic response is based on the photoinactivation of IAA and that in both the acting sensitizer is Rfl, Reinert in the first place explains why the carotene-free base of the coleoptiles still gives normal light-growth reactions. The failure of

unilateral illumination of the base to produce phototropic curvatures is attributed to the considerable transparence of the organ which prevents the establishment of a sufficiently steep transversal gradient of brightness. On the other hand, the high phototropic sensitivity of the tip is explained by the optical filter action of the carotene present which secures the necessary light absorption across the organ, thus protecting the auxin on the posterior side from destruction. Since the difference in brightness between both flanks is mainly controlled by the internal carotene screen, then the phototropic action spectrum must be determined by the double-peaked absorption curve of the carotene. To complete the evidence it has yet to be shown that the action spectrum for the light-growth reaction in the carotene-free base actually corresponds with the single-peaked absorption curve of Rfl.

Reinert's very stimulating argumentation rejects, perhaps too summarily, the possibility of a light-induced transverse auxin migration which, indeed, "has not yet been unequivocally demonstrated" [cf. Galston (13)]. Setting aside this factor of uncertainty, the major obstacle has now been removed which hitherto stood in the way of a satisfactory application of Blaauw's theory to the phototropism of the *Avena* coleoptile.

Further support for the presumption that the actual sensitizer in the phototropic reaction is not the carotene, was obtained from experiments with *Phycomyces* (30). A strain of this fungus produces carotene only when cultured on a medium containing glucose, but none if glucose is replaced by lactate. The sporangiophores of such a carotene-free variety appeared to have retained their full phototropic responsiveness; they seemed, indeed, even more sensitive than normal ones. This last result is ostensibly in agreement with the particular optical situation in such organs in which, owing to a lens effect, the light-growth reaction is transferred from the front to the rear. Therefore, the presence of a protective carotene filter in them could only reduce the transversal light gradient and, if the actual sensitizer were Rfl, would thus impede the development of a phototropic curvature. Later it will be shown that this deduction may possibly be fallacious.

Turning now to the phototropic response of dicotyledonous leaves, the question of the perception of the stimulus poses itself again, but from a different angle. Here the main problem is that of the respective parts played by blade and petiole in the whole chain of reactions. Brauner & Vardar (8) examined the situation in the peltate leaf of *Tropaeolum*. Lateral illumination of the whole leaf produces a strong positive curvature of the stalk. Removal of the lamina drastically reduces the extent of the reaction. On the other hand, screening of the blade with black paper hardly impairs the reactiveness of the petiole, while the opposite arrangement, lateral illumination of the lamina with the petiole shaded, produces no curvature at all. Thus, it appears that the light stimulus can be perceived by the petiole only; full activity, however, depends essentially on the presence of the blade. These relations were clarified by showing that decapitated petioles supplied with 10 p.p.m. IAA solution from a capillary fixed to their distal ends became al-

most completely reactive. Thus, in this reaction, the blade appears to act solely as a natural source of auxin for the petiole, enabling the petiole to carry out growth curvatures.

According to Kuse (20) similar conditions seem also to prevail in the leaves of *Ipomoea batatas*. This worker furthermore could show that tri-iodobenzoic acid (TIBA) in lanolin paste, applied as a narrow ring around the middle part of the petiole, effectively blocks the basipetal progress of phototropic or geotropic curvatures beyond this point. As TIBA is known to act as an IAA antagonist, one can imagine that by penetrating into the path of auxin migration it establishes a road block for the IAA coming from the blade, thus preventing any growth reactions in the lower half of the petiole.

However, the mechanism just outlined does not seem to be the only way in which blade and stalk co-operate in producing phototropic responses. The well-known fact that asymmetric shading of the lamina often causes the petiole to move the lamina into the light, indicates that, under these circumstances, the blade actually can function as a perceptive organ for the light "stimulus." The conditions necessary are, first, that definite areas of the blade supply definite sectors of the stalk with auxin, and second, that the amount of that supply can be locally controlled in the blade itself by light, e.g., by Rfl-sensitized photolysis. Such a system was reconstructed by the present writer (7) using young seedlings of *Helianthus annuus*. Both cotyledons were amputated, and upon their wedge-shaped stumps capillaries were fixed, filled with a Rfl/IAA mixture. Subsequent illumination of one of the capillaries soon produced a distinct growth curvature in the hypocotyl directed towards the exposed tube.

In this experiment the anatomical situation was particularly favorable since the conducting connections between the midrib of the cotyledon and the respective flank of the hypocotyl are easily traced and remain strictly localized, thus fulfilling the main structural requirement. However, corresponding conditions often seem to prevail in individual foliage leaves if the vascular supply of certain lateral areas of the blade enter the petiole independently of the midrib. In such cases, asymmetric illumination of the blade can be expected to cause a corresponding asymmetry of growth in the petiole, resulting in a lateral movement of the whole leaf away from the shade.

The particular optical situation in the *Phycomyces* sporangiophore has been previously touched upon. Banbury (3, 4) reports several new facts about the mechanism of growth and of the phototropic response in these organs. After discussing the distribution of light in the unilaterally illuminated sporangiophore the writer first tries to verify a statement made by Heuckel (18) who claimed that with one-sided irradiation, the protoplasm on the more rapidly growing far side becomes optically denser. No evidence corroborating this observation could be obtained. Then Buder's famous experiments (9) on the inversion of the phototropic reaction in sporangiophores submerged in liquid paraffin were repeated and fully confirmed. To test the possibility that the effect might be due to the unavoidable exclusion of free oxygen

rather than to the inversion of the optical situation, Banbury examined the behavior of his cultures under strictly anaerobic conditions (N_2 atmosphere). Unilateral illumination failed to produce negative curvatures or any other kind, because the complete lack of O_2 had brought all growth to a virtual standstill. From these results Banbury concluded that Buder's obvious interpretation of his (Buder's) experiments was correct. Unfortunately, the author takes no account of Ziegler's work (37) on the same question which shows the matter in a rather different light. We shall return to this paper presently. However, Banbury's positive contribution is the successful demonstration that tangential illumination of the sporangiophore with a narrow light beam causes a more rapid growth of the flank "grazed" by the rays, resulting in a curvature out of the beam. As under these conditions only the directly exposed side could absorb light, while the rest of the hypha remained in darkness, the result proves the reactive independence of the opposite sides of the cell and thus confirms the basic assumption of Blaauw's theory for this object.

In his second paper (4) Banbury deals with the problem of the regulation of growth in the sporangiophore. Years ago, Kögl & Verkaaik (19) were able to demonstrate the presence of comparatively large amounts of heteroauxin in *Phycomyces*. Still an open question, however, is whether the IAA has any effect upon the growth of chitin-walled fungus hyphae. The author tries to decide the matter by the use of the lanolin paste method. By means of a micromanipulator small patches of paste containing from 10 to 10,000 μg/ml. IAA were unilaterally applied to sporangiophores just below the sporangium. None of the concentrations used produced any significant curvature reaction. On the other hand, remarkable results were obtained with griseofulvin, the metabolic product of another fungus, *Penicillium janczewskii*. When administered in the same way as IAA, it causes a sharp bending of the sporangiophore away from the paste, thus showing a local increase of growth at the point of application. Griseofulvin can therefore be considered as a "myco-auxin," acting upon chitin-walled hyphae. However, as its natural occurrence outside the genus *Penicillium* seems to be very doubtful, the bearing of this observation on the growth physiology of *Phycomyces* is rather limited. Moreover, griseofulvin is known to be a very stable substance whose photo-inactivation by any of the sensitizers in question seems most improbable. Thus, even if its occurrence should be discovered in *Phycomyces* also, its participation in any light growth reaction appears rather questionable.

To return once again to the problematic function of IAA in the sporangiophore, the reviewer cannot regard the evidence available as sufficient to justify discarding altogether the possibility of a growth promoting effect. One can easily imagine that the hormone is without additional effect in concentrations in excess of a certain level. Now, if the considerable amount of IAA normally present in the sporangiophore is already in excess, a further increase of this concentration by external supply cannot be expected to produce any visible effect. This may have been the situation in Banbury's

experiments. As to the question of a participation of IAA in the phototropic reaction, the crux of the matter lies in the fact that in *Phycomyces* the light-growth response is positive. Thus, photolysis can explain the situation only if one admits the natural IAA concentration in the organ to be supraoptimal, so that its reduction must, within limits, result in a promotion of growth. This, incidentally, would be the only situation which could account for a Rfl/IAA reaction system being involved in the primary light effect as postulated by Reinert (30). In order to conform to both Banbury's and Reinert's observations, the initial trend of the reaction curve of IAA in *Phycomyces* must therefore be presumed to show a marked peak in the range of subnormal concentrations from which it then drops to the lower, constant level as suggested above. As matters stand, a definite decision on the whole question can thus be expected only from an experimental verification of the actual course of this curve.

Buder's work on *Phycomyces* was also the starting point for another recent study of the mechanism of phototropism. Ziegler (37) critically tested the liquid paraffin experiments by comparing in the same medium the behavior of other plant organs differing from the sporangiophore in their optical qualities. The inversion of the phototropic response by liquid paraffin is not confined to very transparent objects like the *Phycomyces* sporangiophore or the rhizoids of some *Hepaticae* (11), but also occurs in rather opaque hypocotyls like those of *Helianthus* and in the *Avena* coleoptile, where a lens effect is out of the question even in air. Since in these organs, unilateral illumination must thus produce the stronger light-growth reaction in the anterior flank, Buder's optical theory is not applicable to this new situation. Ziegler, therefore, proposes the alternative view that liquid paraffin, owing to its dielectric properties, produces some change in the surface charge of the tissue, thus modifying the primary photoelectric reaction in the system.

To test this possibility, the effect of several fluorescent dyestuffs (eosin, fluorescein, oxypyren, etc.) upon the phototropism of various roots and shoots was examined in aqueous solutions. With roots a reduction of the growth rate was always observed, producing positive "phototropic" curvatures in normally aphototropic types as well as in the negatively phototropic ones. In coleoptiles and in several hypocotyls, however, positive photo-growth reactions could be induced, leading to negative phototropic curvatures. Experiments with *Phycomyces*, unfortunately, remained inconclusive. Unexpectedly, both roots and shoots reverted to their normal tropistic behavior if illuminated in air after the dye-treatment. Among the possible causes of the inversion effect, the following ones were excluded: lack of free O_2, an optical effect of the dye bath, changes in pH, and definite structural peculiarities of the active dye molecules. Ziegler, therefore, arrives at the conclusion that the factor actually responsible must be a basic photoelectric effect modifying the membrane charge at the surface of the irradiated organ.

He starts on the hypothesis that extension growth is promoted by a rise of charge in the structure concerned which increases the mutual repulsion of

the micellae. In positively phototropic organs the sign of the original charge is assumed to be negative. On illumination in air presumably some sort of Hall-wachs effect develops in the membrane, reducing its negative potential and, thus, causing it to contract. Irradiation in the dye solution, however, may be expected to produce a change in the opposite direction by transferring electrons from the dye molecule to the membrane. The resulting increase in negative charge, then, is believed to bring about an extension of the wall. In this way the author tries to explain how, in positively phototropic organs, the reaction is reversed in the presence of both fluorescent dyestuffs and liquid paraffin. The same photoelectric activity is tacitly ascribed to the paraffin as to the dyestuffs. In negatively phototropic organs, on the other hand, the original charge of the membranes is presumed to be positive, so that the identical photoelectrical effect (loss of electrons!) would result in a positive light-growth reaction.

Interesting as the experimental findings of this paper are, the hypotheses advanced do not always stand the test. Particularly, Ziegler's re-interpretation of the basic *Phycomyces* experiment leads to inconsistencies which cannot be removed without ignoring the empirical facts. To explain the positive light-growth reaction in photoelectrical terms, one has to attribute a positive charge to the normal chitin wall. This contention, though somewhat less improbable with chitin than if applied to cellulose-walled cells, still needs experimental confirmation under biological conditions of pH.

As to the optical aspect of the problem, one cannot eliminate the fact that, with unilateral illumination, submersion in liquid paraffin does shift the field of the maximal photoeffect from the rear to the front half of the sporangiophore. If the paraffin medium simultaneously reverses the sign of the photoelectric reaction also, then the direction of the phototropic movement should remain unchanged, which is certainly not the case. This criticism obviously does not apply to the interpretation of the behavior of opaque organs in which liquid paraffin fails to reverse the distribution of light.

Here also an alternative explanation should be considered which does not altogether disregard the auxin mechanism of the light reaction. The extreme lack of O_2 which eventually leads to a complete inhibition of growth, can hardly be without effect on the responsiveness of the tissue even at an earlier stage. If the developing asphyxia should affect the auxin sensitivity of the organ in such a way that the concentration of the hormone present in darkness becomes supraoptimal, the photolytic reduction of the concentration could be expected to cause a positive light-growth reaction. Thus, exclusion of O_2 would condition such organs to the same type of light response which, in *Phycomyces*, prevails even with normal respiration.

The importance of the auxin action curve for the determination of the sign of tropistic movements was convincingly shown by Pilet (24) in his study of the phototropism of lentil roots. The direction of the reaction in this root varies with its age, changing from positive in young specimens to negative in older ones. The actual inversion starts when the root has reached a

length of about 20 mm. and is confined to the extreme tip region. In longer roots the negative movement gradually spreads towards the base. For the basal experiments ultraviolet light was used, but similar results were also obtained with blue and white light, the sequence of effectiveness being ultra-violet$\gg$blue$>$white. Unfortunately, the intensities of both white and mono-chromatic light are given in lux which makes any quantitative comparison rather doubtful. Pilet tries to explain the inversion of phototropism on the ground of his earlier analysis of the age-dependence of the auxin content in lentil roots. He found that the hormone concentration rises considerably during growth, being about 1000 times higher in roots 22 mm. long than in very young ones 8 mm. in length. In the earlier paper he also showed that IAA solutions from 10^{-12} M up to 10^{-6} M stimulate growth in the root while concentrations above 10^{-5} M inhibit it. From these facts Pilet infers that in young roots the natural auxin content is suboptimal, but that it is supraoptimal in old ones. Since unilateral light lowers the auxin level in the illuminated flank, there must be positive curvatures in young roots and negative tropism in old roots. In support of this view experiments are reported in which the auxin content of young roots was raised artificially by growing them in IAA solutions of various strengths. With increasing external concentrations of IAA the positive phototropic tendency gradually disappeared and was finally replaced by a negative movement. However, this last result can be accepted only with caution, because the IAA concentrations producing a definite inversion ($10^{-4}M$ and $10^{-2}M$!) are already dangerously high if not actually poisonous. Thus, their effect on living roots can hardly be accepted as physiological. Apart from this flaw Pilet's arguments are quite convincing. Nevertheless, the reviewer feels that the alternative assumption of a change in auxin sensitivity in the ageing root should also be given due consideration. The importance of this rather neglected point was already stressed in the discussion of Ziegler's work.

Backus & Schrank (2) continued their effort to reveal the correlation between light-induced electrical potential differences and phototropic reaction. In contrast to the previously discussed papers, the present work puts the main emphasis on the possibility of a transverse redistribution of auxin by the light stimulus, and questions the postulate that such a translocation could be attributed to electric forces. The authors start on the consideration that cataphoretic auxin transport would shift the hormone to the positive pole of a transverse electric field. Now, if the primary cause of a phototropic curvature really is an electric polarization of the stimulated organ, then the surface of its more strongly elongating flank should be positively charged. An object particularly suited for a decision of this question seemed to be the *Avena* coleoptile in which, according to the intensity of the stimulus, positive or negative curvatures can be induced. Two nonpolarizable electrodes were placed at the opposite sides of the coleoptile below the tip, and a third at the base. Shive's solution was used as a contact liquid. On each flank the potential difference between upper and lower contact was measured, first in dark-

ness and subsequently after illumination of one of the sides with light quantities producing positive or negative curvatures [200 meter-candle seconds (mcs.) and 31,200 mcs., respectively]. On application of 200 mcs. both apical electrodes became strongly negative with respect to the base, but that on the lighted side more than that on the dark side. Thus, in the unilaterally illuminated coleoptile a transverse potential difference of 15 mv. (maximum) resulted, having the positive pole on the shaded side. Since at the same time a distinct positive curvature developed, this result agrees with the original conception. Illumination with 31,200 mcs. at first also raises the negative potential in the apex; after 20 min., however, the lighted side starts losing most of its charge while the shaded side maintains it. The transverse potential difference which results has, therefore, its positive pole now on the lighted flank. As the phototropic reaction this time is slightly negative, the simultaneous reversal of the electric polarity once again conforms with the author's view.

In another paper Schrank (33) approaches the question from a different angle. If the existence of an inherent electric polarity, he argues, is an essential requirement for the realization of tropistic curvatures, then it should be possible to inhibit the reaction by short-circuiting this potential difference. For the verification of this proposition, cut coleoptiles were used from which the primary leaf was removed. Then the cavity was filled with electrolyte solutions of various strengths (Shive's mixture $\times\frac{1}{2}$, $\times 1$, and $\times 2$) and, for comparison, with distilled water or with a glycerol solution as an osmotically equivalent non-electrolyte. The specimens thus prepared were unilaterally illuminated with 200 mcs. and the resulting curvatures measured. It then appeared that air-filled controls gave the strongest final reaction, whereas distilled water and all three electrolyte concentrations reduced it drastically. Glycerol, on the other hand, after an initial acceleration, brought the movement to a premature standstill. From these rather confusing results Schrank concludes that the observed inhibition of the reaction by the electrolyte solutions must be due to a shunting of the inherent electric field whose indispensability he thus believes to be proved. Though the reviewer fully shares the author's view that the part played by electrical factors in tropistic reactions deserves very careful consideration, he does not yet feel entirely convinced that the evidence presented in the last two papers is fully conclusive. As previous experience has made it very probable that photoelectric effects of the type described are the outcome of photo-permeability reactions interfering with preexisting diffusion potentials, it first has to be proved that the secondary electrical effect rather than the primary permeability change initiates the tropistic reaction. Particularly in the "first" negative curvature the participation of a phototurgor reaction cannot entirely be disregarded. In the shunting experiments, on the other hand, one must ask to what extent the inevitable change of the optical situation, caused by the presence of a liquid in the cavity of the coleoptile, may have interfered with the phototropic reaction. The fact that distilled water was found to inhibit the response

no less than did normal Shive's solution (specific conductances 9.3×10^{-6} and 4.2×10^{-3}, respectively!) can, in any case, be interpreted more easily in optical than in electrical terms.

GEOTROPISM

Since a direct chemical effect of gravitation upon auxin metabolism is hardly conceivable yet, the translocation problem has, naturally, been much more in the foreground in recent geotropic research. Schrank in his paper quoted above (33) also deals with the dependence of the geotropic response on the existence of an electric polarization in the stimulated organ. The principle of the method employed was the same as in the corresponding phototropic experiments. Cut *Avena* coleoptiles, either left filled with air or injected with various concentrations of Shive's solution, were placed horizontally and the resulting geotropic upward curvature observed for 1 hr. For comparison, samples filled with glycerol solution, osmotically equivalent to the highest electrolyte concentration, were included in the set. This time much clearer results were obtained than with phototropic stimulation: coleoptiles containing air and those filled with glycerol now reacted almost identically, both giving the strongest response in the whole series. On the other hand, all tested gradations of Shive's solution reduced the reaction, but this time the inhibition effect increased in regular proportion with the concentration. Conditions in these experiments were, obviously, less complex than in the phototropic series, because the optical influence of the liquids in the cavity now remained ineffective.

In a recent paper (34) Schrank continued his analysis. By comparing the effects of several one-salt solutions in different concentrations he tried to find out whether the observed inhibitions were directly determined by the specific conductance of the electrolytes used. The relative effectiveness of the three alkali chlorides LiCl, NaCl, and KCl did not show the expected simple correlation. Although within the concentration series of each of these salts the inhibition showed the same regular rise as it did with Shive's solution, the effects in equimolar gradations did not reflect the marked differences in their specific conductances. Particularly, lithium chloride which, in equivalent concentration, possesses the poorest conductivity among the three salts, produced the most vigorous inhibition. It, therefore, had to be presumed that a specific ion-effect interferes with the basic resulting of shunting. The nature of this secondary influence is not discussed, however. In the present writer's view chiefly two such effects have to be considered which may be no less important than the shunting factor: (*a*) the lyotropic effect of the ions added which can be expected to alter the permeability of the coleoptile tissue, and (*b*) a direct interference of the solutions injected into the cavity with the "physical" geo-electric effect, developing in the horizontally placed organ. It is also quite possible that a correlation exist between *a* and *b*, because the development of a geo-electric potential difference is known to depend on the ion permeability of the membranes concerned. The basic question, how the establishment of a transverse potential difference eventually leads to the

translocation of auxin is still left unanswered by Schrank, though he seems to consider some direct cataphoretic mechanism.

A different view is held by Bünning & Glatzle (10) who suggest that the primary action of gravitation, whether it is a translocation of statolith starch or the geo-electric effect, brings about the eventual displacement of the hormone; not directly, but through the intervention of some intermediate reaction as yet unknown. The authors consider the possibility that the stimulus of gravitation may, as is often assumed for mechanical stimuli, first release an "all-or-none" reaction which, in its turn, activates the subsequent steps of the process. In such a case, the stimulated cells could be expected to pass through a refractory period during which the continuation of the stimulus would remain ineffective. If this view describes the situation correctly, a given amount of geotropic stimulus should prove more effective if presented in instalments. Roots of *Lepidium* and *Avena* coleoptiles were geotropically stimulated by placing them horizontally for 5 min. This amount of stimulus was given either continuously or in two parts of 2.5 min. each, separated by various periods of horizontal rotation. In both cases the reactions were allowed to develop on the klinostat. The results obtained seem to bear our Bünning's thesis. Intercalation of a stimulus-free period into the exposure time caused a distinct increase of the final response, the optimal length of the interval being 6 min. for *Lepidium* and 30 min. for *Avena*. A further prolongation, however, diminishes the effect in both cases.

Bünning's interpretation of the situation is that the individual cells of the sensitive organ react according to the all-or-none rule, but their threshold of irritability differs slightly. Thus, after the first period of stimulation a certain number of cells will have reacted and consequently have become refractory. During the subsequent relaxation period these cells regain their irritability, so that after a suitable interval the second half of the stimulus again meets the original number of reactive cells. On the other hand, with uninterrupted exposure to the same total amount of stimulus the number of refractory cells is steadily growing. Now, if the reactions of the individual cells actually are of the all-or-none type, it follows that the tropistic behavior of the organ as a whole must be the expression of a summation of such single reactions, transforming the absolute refractory period into a relative one. This may explain why, in such systems, even continuous exposure still increases the response during the second half-time. In a recent review Guttenberg (17) objects to this concept on the grounds that if the all-or-none rule were valid, then the second stimulus reaching the organ after termination of the refractory period would find it back in its original condition. Thus, only an exact repetition of the first response could be expected, excluding any summation effect. In the present reviewer's opinion this criticism disregards one important factor. Though during the relaxation period the original level of irritability may be completely regained, the secondary changes initiated by the first stimulus will still persist and be able to sum up with identical changes resulting from the second stimulation.

Ziegler (38) studied some of the metabolic changes which accompany the

geotropic reaction. Starting with statements by earlier workers [Metzner (21), Warner (35)] he re-examines the question of sugar distribution in geotropically stimulated organs, using a new electrotitrimetric method. The experimental objects were stems of *Helianthus* and *Bryophyllum*. First, the course of the geotropic curvature was registered. In *Helianthus* the movement became visible after 45 min., and after 14 hr. the vertical position was reached. *Bryophyllum* reacted more reluctantly, the corresponding times being 6 hr. and 24 hr. Parallel sugar determinations confirmed earlier findings that during the geotropic movement the lower half of the stem contains distinctly more reducing saccharides than the upper half. The development of this difference in both plants coincided with the appearance of the curvature. In *Helianthus* it reached its maximal value (40 per cent) when the movement was just completed, and was followed by a gradual equalization of the concentrations. In *Bryophyllum*, however, the parallelism was far less conspicuous. Here the maximal concentration difference (again 40 per cent) appeared 4 days after the end of the geotropic movement. It took one more week until the now vertical stem became completely depolarized.

In a third series, the respiration of the stem during its geotropic movement was measured by determining the oxygen consumption of its separated halves in the Warburg apparatus. Simultaneously with the beginning of the curvature and of the unilateral increase of the sugar concentration, the intensity of respiration in the lower half started rising above that in the upper half. The trend of this change also followed a maximum curve running approximately parallel with that of the sugar accumulation. It, therefore, was obvious to expect a causal relation between both phenomena. However, the following experiment showed the fallacy of this assumption. By suspending stem tissue in glucose solution the respiration rate can be increased to a maximum, attained at a sugar concentration of 1.5 per cent. Now, if the upper and the lower halves of geotropically stimulated stems are transferred to such a glucose solution, the difference between their respiration intensities should disappear if it be due to the sugar level. Actually, however, the disparity, though somewhat reduced, did persist even in 2 per cent glucose. In the absence of more satisfactory evidence Ziegler suggests that the intensification of repiration in the lower half may be induced by the translocation into this half of IAA which is known to stimulate biological oxidations. A corresponding mechanism is also considered for the explanation of the unilateral accumulation of sugar. In this case the effect is referred to a hypothetic downward migration of amylase. Incidentally, it may be remarked that the trend of the observed changes in sugar concentration excludes the possibility of their direct osmotic effect upon the geotropic movement.

Pilet and his associates (25 to 29) approach the question of carbohydrate transformation in the geotropic reaction from a different angle. They start on the assumption that some correlation may exist between the function of the statolith starch and that of auxin. The main experiments were carried out with lentil roots. It appeared that the amount of statoliths, after

an initial rise, drastically decreases as the organ grows older. Since simultaneously the auxin content in the root rises, the possibility of an amylolytic effect of auxin is considered. This view finds support in the observation that treatment of roots with $10^{-6}M$ IAA solution markedly reduces their starch content, particularly in older ones. Similar results were also obtained with shoots of *Cirsium arvense* on application of another growth substance, 2,4-D (dichlorophenoxyacetic acid). In further experiments, the hydrolyzing effect of IAA was more closely examined. It appeared that buffered starch solutions were not attacked by IAA *in vitro*. On the other hand, tissue brei of lentil roots added to starch powder and soaked with $10^{-4}M$ IAA solution did produce a slight disintegration of the starch. Unfortunately, no control test was made with auxin-free brei, which makes it rather difficult to judge to what extent this last result was due to a possible amylolytic action of the brei itself. In a third experiment, the saccharification of starch solution by amylase contained in the culture medium of a fungus, *Coniella diplodiella*, was measured in the absence and in the presence of IAA. No influence of the auxin on the rate of starch decomposition could be observed. From the sum of these observations Pilet concludes that IAA *in vitro* has no influence on the activity of amylase nor does it attack starch directly. But, as it does induce the disappearance of statoliths in living cells, an indirect activation of hydrolysis may take place, e.g., through some enzyme system mediating between auxin and amylase. For more comprehensive information on this question the reader is referred to a recent paper by Anker (1).

After thus having prepared the ground, Pilet (25) tries to arrive at a synthesis of the statolith and the auxin theory of geotropism. He begins with the fact that young roots of *Lens* contain less auxin than old ones. Hence, it is assumed (though not proved) that in the former the auxin concentration is suboptimal, in the latter, supraoptimal. Accumulation in the lower half of the horizontally placed organ could, therefore, be expected to produce a positive curvature only in older roots, while in young roots the reaction should be negative, which happens but very rarely. Thus, with the auxin level suboptimal positive geotropism cannot be explained satisfactorily on the basis of a redistribution of the hormone. Pilet then takes recourse in the statolith theory. He believes that translocation of the starch grains, by some mechanism yet unknown, raises the sugar concentration in the upper part of each acting statocyst. The resulting osmotic polarization then is supposed to produce an augmentation of the osmotic value and, subsequently, of turgescence in the entire upper half of the organ, thus initiating the downward movement.

The experimental evidence upon which this hypothesis is based looks rather scanty, and the reviewer believes that, before elaborating the details, it would have been more profitable to put the basic contention to the test. The observation of a low auxin concentration in young roots is not proof in itself that this low level is bound to be suboptimal; it seems quite possible that the deficiency in quantity may be physiologically compensated for by a

correspondingly higher auxin-sensitivity of the young tissue. So, the whole question will inevitably remain suspended until the concentration/action curve of the growth substance has been reliably established for roots of different ages.

More instructive is another paper by the same author (23) in which the geotropic behavior of the stamens of *Hosta caerulea* (Hemerocallideae) is examined. In the closed buds the stamens are positively geotropic; after the opening of the flower, however, their geotropism changes to negative. Pilet starts on his customary working hypothesis that such a reversal may be induced by a corresponding change in the auxin level which, in this case, may be brought about by the exposure of the stamina to light when the flower opens. Both assumptions could be verified. Using the *Avena* test Pilet could show that in the bud the auxin level in the stamina corresponded to $10^{-4}M$ IAA, whereas in the open flower it had dropped to $10^{-12}M$. Experiments were devised to reveal the effect of light on the behavior of the stamens. In one, buds were left to open in the dark; in another, very young stamens were exposed to daylight by removing the covering tepals. In the dark the stamens retained their positive geotropism even in the mature stage; in the light, they reacted negatively from the outset. In a third experiment buds were injected with potassium indoleacetate solutions of various strengths two hours before opening. This treatment prevented the inversion of geotropism on the subsequent exposure to light, the stamina thus retaining their positive behavior as they did in the dark (optimal auxin concentrations were 10^{-7}– $10^{-9}M$).

Pilet's leading idea has much in common with Geiger-Huber's conception of the determination of the direction of geotropism in stem and root. That author showed in an earlier paper (14) that hypocotyls of *Cucurbita* could be induced to invert their normal negative geotropism into positive by a treatment with supraoptimal IAA doses. However, differing from Pilet, Geiger-Huber does not overlook the equal importance of variations in auxin sensitivity which, in many cases, seem to be as determinant for the direction of the response as is the concentration factor.

Some recent papers consider the question of the perception of the geotropic stimulus by dicotyledonous leaves. In a study, briefly discussed in the section on phototropism, Brauner & Vardar (8) examined the geotropic behavior of the *Tropaeolum* leaf. Petioles of intact leaves, fixed horizontally with their ventral flanks upward, rise in 6 hr. by 40.2°. Removal of the blade reduces this response to 23.4°. However, if the decapitated petiole is supplied with 10 p.p.m. IAA solution from its distal end, not only is the reactivity restored, but appears even enhanced by 30.1 per cent above the normal value. From this observation it can be concluded that in the *Tropaeolum* leaf the geotropic stimulus, too, is perceived by the petiole only, and that the blade merely serves as a source of auxin, enabling the stalk to maintain its growth. In this context it is of some interest that decapitation diminishes the geotropic reactivity of the petiole much less than its phototropic responsiveness.

This fact suggests that the phototropic mechanism requires a greater supply of auxin than does the geotropic response.

Similar conditions were later found by Gessner & Weinfurter (16) in the *Nymphaea* leaf. The starting point for their study was the old observation that cut leaves left floating upon water in a normal position curve their petioles upwards in a direction invariably away from the tip of the blade. This movement whose geotropic background was previously recognized by Germ (15) also includes a strong hyponastic component. Gessner and his colleague were able to show that the reactivity of the petiole depends on the presence of the blade in a way which strongly resembles that of *Tropaeolum*. Decapitated stalks fail to curve, but if the cut-off blade is placed back on the stump and the wound surfaces are brought in direct contact, the petiole reacts as before. Furthermore, the hyponastic tendency of the stalk also is determined by the blade. If cut petioles are joined to their blades with their sides inverted, the ensuing curvature is again in the direction away from the distal end of the lamina, regardless of the morphological dorsiventrality of the stalk. From this result it is concluded that the essential factor determining the nastic movement must be an asymmetry in the auxin supply from the blade, probably due to the larger surface of its distal as compared with its proximal half.

Nevertheless, another factor also participates in the causation of the normal nastic response: a marked difference in the auxin sensitivity between the dorsal and ventral flank of the petiole. In Gessner's experiment this physiological dorsiventrality may have been concealed by the very asymmetric auxin supply from the blade. However, on symmetric administration of identical auxin concentrations to the opposite flanks it shows itself very clearly in the appearance of a hyponastic curvature, as could be demonstrated by Vardar in unpublished experiments. A similar asymmetry, though in the opposite sense, also prevails in the *Tropaeolum* petiole, giving rise, on symmetric auxin supply, to an epinastic movement. It is obvious that this nastic factor must cause a corresponding asymmetry of the tropistic responsiveness, as was indeed shown by Brauner & Vardar (8) with the photo- and geotropism of *Tropaeolum*, and by Gessner & Weinfurter (16) with the geotropism of *Nymphaea*.

The behavior in the gravitational field of physiologically dorsiventral organs can, in principle at least, be traced back to an interference between an orthogeotropic and a nastic component, resulting in a position of rest at definite angles with the vertical. Much more difficult, however, is an explanation of the plagiotropism or diageotropism in such organs which show no sign of a physiological asymmetry. Bennet-Clark & Ball (5) have studied the question with the rhizome of *Aegopodium podagraria* which, in darkness, maintains a roughly horizontal direction of growth. They first observed that the orientation of this organ proves to be extremely sensitive to light which, regardless of its direction of incidence, evokes positive geotropic curvatures. This effect, obviously tonic in nature, not only was produced by white light,

but most surprisingly even by weak red darkroom illumination of as short as 30 sec. duration. Nothing definite can be stated yet concerning the nature of this interference, but a certain analogy with the effect of small light doses on photoperiodic phenomena is quite obvious. To avoid such light-induced changes of the geotonus all recordings of the normal geotropic dark responses had to be made photographically, using infrared radiation to which the rhizome proved to be indifferent. If the organ is inverted by turning it through 180°, first a vigorous upward curvature develops, lasting about 2 hr. Then the rhizome slowly returns, with damped oscillations, back to the inverted horizontal position. The same initial reaction which the authors term "displacement effect," can also be evoked by any other displacement (oblique, vertical), the flank of the organ which was uppermost in the original position invariably becoming convex. In the subsequent phase the direction of the movement is not definitely fixed: it may be a continuation of the first curvature or its reversal. In any case, however, one of the two possible horizontal positions is eventually attained.

While light upsets the diageotropic dark equilibrium in the rhizome by evoking positive geotropism, another agent, viz., augmentation of the CO_2 concentration in the surrounding atmosphere produces the opposite effect, causing the organ to curve upwards. It is obvious that this antagonism may play a part in maintaining the rhizome at a definite level below the surface of the soil.

As a basis for a general interpretation of diageotropism in radial organs the following hypothesis is suggested. Since, in the absence of any inherent dorsiventrality horizontal growth cannot be explained by an accumulation of one single growth hormone, it had to be assumed that together with the auxin a second hormone is translocated downwards, eventually counterbalancing the growth promotion in the lower flank. The postulated substance thus has to possess the qualities of an "antiauxin." Though sufficient in itself to account for the maintenance of horizontal growth, this simple assumption does not explain the characteristic first phase of the growth movement following any displacement of the rhizome. As this initial reaction always appears as an acceleration of elongation in the flank which was uppermost in the original horizontal position, it must further be assumed that both hormones differ in their mobility, the auxin itself being more rapidly translocated than the antiauxin. According to this view, on inverting the rhizome, first the auxin would reach the lower half, and, not being simultaneously accompanied by the antiauxin, could promote growth there unimpeded. However, the antiauxin would gradually follow and, eventually, re-establish the original equilibrium. As an additional factor the authors also consider the longitudinal component of gravitation ("Längskraft") which, conformable to earlier experience with other stems, was found to enhance the longitudinal growth of the rhizome in a vertical downward position, and to retard it in the opposite, upward position. In order to interpret this phenomenon on a hormonal basis it is assumed that this "Längskraft"

interferes with the normal longitudinal auxin transport by accelerating or impeding it, according to the direction of its activity, whether basipetal or acropetal. Retardation of the flow should tend to pile up auxin in the elongation region, acceleration having the opposite result. If, in the oblique position the growth hormone accumulates on the lower side of the rhizome, the longitudinal force would chiefly affect this flank, inducing the organ to return to the horizontal plane. However, in this conception the action of the antiauxin must also be included as warranting symmetry of growth in the horizontal equilibrium position.

For reasons not particularly explained, the writers believe that the growth hormone subject to the influence of the "Längskraft" is probably not identical with that responsible for the primary displacement effect. As they see in it the agent of the final adjustment, they term it "diatropic auxin" to distinguish it from the other, nonspecified growth promoter. This differentiation comes somewhat unexpectedly, because the same essential qualities are attributed to both hormones, viz., the faculty for promoting longitudinal growth, the tendency to accumulate on the lower side of the rhizome, and the susceptibility to the inhibitory effect of the antiauxin. In the reviewer's opinion it would, therefore, be preferable to drop this distinction until the presence of two auxins identical in the mentioned qualities, but differing in others, has actually been proved. Apart from this detail, the proposed auxin-antiauxin hypothesis seems to promise a much better understanding of the diageotropic behavior of radial organs than does the older concept of a simultaneous induction of positive and negative geotropism balancing one another. The actual existence of the postulated antagonistic hormone system has meanwhile been confirmed by chromatographic analysis (6). It could be shown that the main growth promoter in the *Aegopodium* rhizome, 3-indole-acetonitrile, is always accompanied by a growth inhibitor as yet unidentified chemically.

THERMONASTY

From earlier work on the effect of temperature on the opening and closing of certain flowers two points have emerged which can be accepted as definitely proved. One of them is that the movement is caused by a differential growth response of the opposite sides of the reacting leaves, and not, as occasionally contended, by reversible turgor reactions; the second, that the difference in response originates from the fact that the two flanks reach their growth optimum at very different temperatures. Wood (36) resumes the question at this point. In order to get a clearer view of the range of the effect of temperature, he first works out from earlier data the temperature coefficients (Q_{10}) of growth for tepals in different conditions. In the "steady state" (flower neither opening nor closing) more or less normal values were found over a range from 5.5 to 26°C.: $Q_{10} = 2$–5. During the opening phase, however, the coefficient rises enormously on the inner side of the leaf: $Q_{10} = 20$–30, while during closing values extreme in the opposite direction are found on the outer flank: $Q_{10} = 0.1$–0.2. These latter values are an expression of the

fact that on this side an increase of growth is provoked by falling temperatures.

The author then reports some interesting findings of his own, working chiefly with *Tulipa*. He first compares the behavior on warming and on cooling of complete tepals with that of isolated inner and outer halves. In intact leaves a rise in temperature from 7° to 17°C. first produces a rapid opening movement which, after 1.5 hr., is followed by a limited return movement leading to the final new equilibrium at the end of the second hour. When the corresponding thermo-growth reactions were measured with separate halves, it appeared that the outer surface remains practically indifferent to the rise in temperature, whereas the inner side almost immediately increases its growth rate. However, this enhancement is only transitory, and after the first half hour both elongation graphs again run parallel. Thus, only the initial, rapid opening movement of the whole tepal can be referred to the individual growth reactions, while its continuation during the next hour and, more particularly, its later reversal remain unexplained. This situation makes it likely that in the intact organ some correlation exists between both halves, a point which would have deserved some consideration in relation to auxin. The respective reactions on cooling from 20° to 10°C. are in better agreement. The closing movement induced in the intact tepal this time takes a continuous course, no "back reaction" interfering. In the isolated inner half the rate of growth is slightly reduced; in the outer half, however, a marked enhancement develops, lasting about 1 hr. In confirmation of earlier statements it could be shown that the tissue actually responsible for the movement is the thin-walled mesophyll, though the epidermis also seems to participate.

As to the mechanism of the response, it could be demonstrated that during the nastic movement neither the osmotic value, the pH nor the permeability of the protoplast showed any significant changes. These observations are in keeping with recent findings by Mückschitz (22). Yet this worker discovered that another plasmatic factor did change in the thermonastic reaction, viz., the viscosity of the protoplast. In hypertonic solutions the cells on the actively elongating side of the tepal always undergo "cramp plasmolysis," those of the opposite, inactive side, convex plasmolysis. Thus, in thermonastically-opened flowers this asymmetry is the reverse of that in closed ones.

The most important part of Wood's paper deals with the interrelation between the thermonastic reaction and the respiratory changes accompanying it. On warming, both O_2 uptake and CO_2 output rise considerably, the Q_{10} values being markedly greater in lower than in higher temperature ranges. The curve of the respiratory rate follows a trend approximatively parallel to that of the opening movement, showing the same "back"-phase after the initial, drastic rise. From measurements with separate halves it can be seen that the inner surface has a distinctly higher level of respiration than the outer side. Anaerobic conditions (N_2 atmosphere) and respiratory inhibitors (sodium azide) prevent both growth and thermonastic response. On the

other hand, sodium citrate, which promotes respiration, also increases growth.

Most interesting is the effect of the CO_2 level in the surrounding atmosphere upon the temperature responsiveness of the tepals. In complete leaves, raising the CO_2 concentration to 10 per cent promotes, at 8°C., the closing movement; at 16°C., the opening reaction. In CO_2 free air growth appears very much reduced. At 8°C., an increase of the CO_2 level first stimulates the outer flank only; as the concentration is increased the inner side gradually catches up, and above 6 per cent CO_2 its growth surpasses that of the outer side. At higher temperatures, however, even low CO_2 concentrations enhance growth only in the inner flank. Thus, the presence of CO_2 drastically modifies the growth/temperature relation: as the CO_2 level increases, the optimum of growth is gradually shifted toward the lower temperatures. While in normal air the inner side shows its maximal elongation between 17° and 25°C., and the outer side between 8° and 15°C., in 2 per cent CO_2 the corresponding ranges are 15°C. and 7.5°C., respectively. The growth promoting effect of CO_2 seems also to be one of the reasons why complete tepals elongate more vigorously than do their separated halves. The air enclosed in the intercellular spaces contains a considerable amount of CO_2 (2 to 5 per cent) which, on cutting, is much reduced by diffusion. This view finds support in the fact that such separated halves, on transference into air containing 2 to 5 per cent CO_2 attain the elongation rate of intact tepals growing in normal air. Nothing much is known as yet about the nature of the growth-promoting effect of CO_2, but Wood made one important observation which may be the starting point for a future analysis: CO_2 added to the atmosphere, far from being fixed by the tissue, enormously enhances its respiration. Considering the close correlation between respiration rate and growth this rise could be the primary cause of the CO_2 growth response.

From all these findings it can be inferred that in the thermonastic reaction at least two factors are involved: (a) a considerable difference between the temperatures at which growth has its optimum in the opposite sides of the tepal, and (b) the high internal CO_2 concentration which "sensitizes" the tissue to temperature changes. The principle of this mechanism bears some formal resemblance to that of some auxin-induced nastic responses where identical hormone concentrations act differently in the opposite halves of the organ. In such systems, too, the direction of movement can be reversed by a uniform change of the intensity of the agent.

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DORMANCY IN WOODY PLANTS[1]

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DEFINITIONS AND DELIMITATION

The term "dormancy" is generally associated with the temporary suspension of visible growth, especially that of buds and seeds, without regard to its cause. The causal factors may be of two kinds. Growth may be stopped because of external conditions, i.e., unfavorable temperature, water supply, etc; this kind of dormancy we shall call "quiescence," as suggested by Meyer & Anderson (87). The second, that dormant state which is caused by internal factors, i.e., a suspension of growth which continues even under favorable external conditions, we shall call in agreement with Chandler (19) "rest." It seems useful to agree on some terminology which differentiates between these concepts, since they describe physiologically different phenomena. Nevertheless, even recent treatises on plant physiology do not distinguish between "dormancy" and "rest."

As was noted above, "dormancy" is generally associated with the suspension of visible growth. One cannot describe this state merely as "a suspension of growth" because even during rest a slow but continuous growth takes place in the bud as was shown by Chandler & Tufts (23), as well as by Bell (2). Therefore, some writers (87) have used the term "sprouting" to designate the inception of visible growth. Nevertheless, cambium growth, which is not visible to the unaided eye, represents the end of dormancy of this tissue, and would have to be mentioned specifically in an all-inclusive definition.

When internal conditions of the bud, causing rest, are due to factors arising outside the organ, the term "correlated inhibition" (25) is used. When these factors arise within the organ, the condition is that usually understood under the term "rest" in its narrower meaning; but it is distinguished only with difficulty from "cyclic growth," the regularly recurring interruptions of growth in evergreens. Though rest of buds is usually associated with deciduous trees, it is not causally related to leaf-fall, since leaf-fall occurs after the tree has entered the rest period. It has been demonstrated repeatedly since Schimper that many deciduous plants, when transferred to a moist, tropical environment, will show the cyclic growth of evergreens. It may be, then, that the interruption of growth in the evergreen fulfills the same function as the rest period in the deciduous tree, although the growth-cycle in deciduous trees may not remain the same when the plant is transferred to evergreen conditions.

Several analogous phenomena can be mentioned in connection with breaking rest in deciduous trees, and the conditions which precede the growth flush in evergreens. The accumulation of starch which precedes the growth flush in citrus is also associated with rest in deciduous trees [James & Stein-

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[1] The survey of the literature pertaining to this review was concluded in July, 1953.

acker (64)]. High temperatures, found by Humphries (63) to be associated with resumption of growth in cacao, are sometimes operative in breaking the rest of deciduous trees. Chandler (20), however, mentions one factor which differs in evergreens and deciduous plants: chilling terminates rest in the latter but does not terminate growth suspension in the evergreen. Cessation of growth in evergreens, therefore, may not be a resting condition. Further work on the relation between cyclic growth and the rest period might prove fruitful.

Correlated inhibition, which ocurs when the growth of a lateral bud is suspended by an inhibiting factor originating in a terminal bud or adjacent leaf, would seem more easily distinguished from rest; still, here too, the distinction is not a sharp one. Thus, it has been said (18, 45) that the removal of an adjacent leaf which causes sprouting of its axillary bud is a breaking of rest. However, it is also well known that substances arising in the leaf may cause a suspension of growth in the axillary bud (50); hence removal of the leaf must be considered to cause the termination of a correlated inhibition. It may be, nevertheless, that the inhibited bud gradually enters rest, and that rest is broken when the leaf is removed; i.e., that rest is broken in the bud by an operation on an organ external to it.

Again, inhibitors are synthesized in the bud-scales of resting buds (59). If such inhibitors importantly affect the rest of the growing point, it is hard to draw a line between "rest" and correlated inhibition, since bud scales, although part of the bud, are external to the growing point which, in a narrower sense, is the organ affected by rest.

The subject of rest, even when limited to its restricted definition, is so large that this discussion must be confined, almost entirely, to the behavior of buds on perennial shoots. Rest in seeds must be excluded because it is more decisively affected than is rest in buds, by certain factors, i.e., structural features, developmental stages in the embryo, and because the physiological responses differ widely in different species. The reader will find this field covered in recent reviews: Porter (100) on seed technology, Evenari (41) on germination inhibitors, Stiles (121) on the effect of light, and Murneek & Whyte (92) on vernalization.

Discussion of rest in bulbs, corms, and tubers must also be excluded, for their large size and other specific structural features in some cases affect respiration and hence rest; and certain physiological responses vary greatly in various species [Blaauw (7)]. Their fundamental processes, however, are similar to those in buds and there is, in fact, little basic difference between the eye of an underground stem and the bud of its above ground counterpart. Because such important contributions to the study of the mechanism of rest have been made in potato by the Boyce Thompson Institute (27), and recently by Hemberg (57, 60), no discussion of this subject can be complete without references to their findings.

DESCRIPTION OF THE REST PHENOMENON

In addition to this review on modern studies of rest in perennial stems, the reader is referred, in the older literature concerning the termination of

rest, to the methods described by Weber (137) and to the concepts discussed by Molisch (90) as well as by Howard (62). More recently, Stoughton (122) described the horticultural aspects,[2] while Chouard (25) has reviewed this subject within a broader frame in the light of modern biochemical advances.

Because of space limitations the writer regrets that he must disregard the question of priority for particular concepts, and must forego complete discussion even of more recent publications.

The entrance into rest is usually preceded by a stage of quiescence (25) either brought about by shorter days, cold, heat, drought, or other conditions unfavorable to growth, or due to correlative inhibition. Quiescence or inhibition go over into "preliminary rest," an early stage during which the dormant bud will no longer grow in response to favorable conditions, but can be easily "forced" by subjection to cold or heat, wounding, treatment with anaesthetics, etc. During "mid-rest," only the most drastic treatments will stimulate a growth response, which will even then be weak as compared with either "preliminary" or the following "after-rest"—two periods having much in common. "After-rest" may be followed by another period of quiescence if growth conditions are unfavorable. It is important to distinguish between stages of rest and of quiescence in reporting experiments on dormancy. In appraising results it will, for instance, make considerable difference whether a treatment has been given to a quiescent bud after rest had ended, thus merely hastening growth, or whether such treatment actually did break the rest. Much of the literature lacks clarity and precision because such distinctions are ignored.

With entry into the dormant state, bud growth is slowed but does not stop completely, even in midrest unless, of course, the temperature falls below minimum growth-requirements. To produce normal growth under favorable conditions, however, it is necessary that rest be "broken" by a certain period of cold during which growth may, in fact, be interrupted. The length of this period, which differs with the variety and with the species as well as with physiological conditions is termed the "chilling requirement." Chandler & Tufts, for instance (23), showed that resting flower buds of peach whose growth had been interrupted during a protracted period of chilling, ultimately overtook the development of buds, kept at higher temperature, whose growth had never ceased.

When the chilling requirement of embryos is not fully satisfied and their rest is thus incompletely broken, the seedlings developing from them exhibit sluggish growth. Such abnormal growth behavior can be normalized by subjecting the seedling to a cold treatment. Flemion & Waterbury (44) have described such peach seedlings whose shortened internodes and reduced leaves resembled a "rosette" which was strongly reminiscent of growth mani-

[2] After this paper was sent to the printer, the writer received the review on Dormancy in Buds of Woody Plants, by J. Doorenbos, *Med. Landbouwhogeschool, Wageningen*, **53**, 1–24 (1953).

festations resulting from interference with the development of auxin, its transfer, or the inhibition of its action.

When rest is insufficiently broken, as occurs frequently in warm climates, bud opening will be irregularly delayed and some will not open at all. Buds may "flare" (66), i.e., they may start to open, but the shoot, failing to develop further, may die. Leaves may be deformed, multiple pistils may appear producing multiple stone fruits (75), the style may fail to develop, and pollen development is likely to be poor.

When the chilling requirement is unsatisfied, abscission of buds or parts therein is frequent. Thus, with pome fruits which have mixed buds, all primordial flowers or part of the cluster may abscise (16) so that the flower bud will produce a reduced cluster or open only into a leafy spur (20). In other cases, e.g., stone-fruits (15) and pistachio (143), the entire bud drops. While warm winters cause delayed opening of buds and the above-mentioned growth-irregularities as well as bud drop, it may not be quite justified to assume that the mechanism which causes bud drop is the same as that which causes the first two phenomena. While it is true that both abcission and delayed opening will be more prevalent after warm winters, the prevalence of bud drop is not always correlated with relative chilling requirement. Thus, Brooks (14) reports that French prune shows a much lighter bud drop than Kelsey plum, although Kelsey is known (109) to have a much shorter chilling requirement as judged by date of blossoming and foliation. A similar lack of correlation can be found in the data reported by Weinberger for peach varieties (e.g., 139). Furthermore, as will be shown in another connection, treatments which break the rest of buds do not affect bud drop. While both irregularities of growth and abscission are certainly the consequence of insufficient termination of rest, nevertheless, there seems to be enough difference between the mechanisms involved to justify a separate investigation of bud drop. Detailed discussion of the horticultural aspects of prolonged rest may be found in the papers by Chandler & Brown (16, 21), by Black (6), as well as by Hill & Campbell (61).

Rest has been found to be accompanied by certain cytological phenomena. It has been found that cells within such tissues as the cambium shrink during rest and that the protoplast develops an opaque appearance and gel-like properties, as compared with its translucent sol state after rest is terminated (124). Breaking the rest in potato was found to be accompanied, also, by an increase in chromosomes and mitochondria (80). Genkelj & Oknina (48) observed, furthermore, that when the cell enters rest the protoplast contracts and assumes a convex shape. Withdrawal from the cell wall results in a rupture of the plasmodesmata. The protoplast covers itself with a visible lipoid layer which may prevent drying out, but would, on the other hand, also reduce the entry of water and solutes into the cell. This isolation of the cell during rest ends with the termination of the rest period, when the protoplast swells and reestablishes plasmodesmic connections (97). Although Meeuse (86) holds that such complete loss of plasmodesmata is rather improbable, the above observations have been repeated by Genkelj & Oknina

(48) on *Pinus, Taxus* and other evergreens as well as on deciduous plants. The separation of the protoplast is most evident in the apple and pear, and less so in the grape vine, but the walnut does not show it at all. This is unexpected in view of the relatively great chilling requirement of *Juglans regia*, as compared with *Vitis vinifera;* because of this the question arises whether protoplast contraction is a direct function of rest or whether it is a corollary of frost resistance, as the authors suggest. When rest was artificially terminated by means of a warm bath, plasmodesmic connections were resumed in the same way as when rest is terminated naturally. Furthermore, the same phenomenon of contraction was observed during the summer rest both of potatoes and of roots of *Taraxacum kok saghyz* [Russian dandelion (112)]; the latter suggests either that the phenomenon is not fundamentally connected with rest, or that some roots may have a true rest period.

Organs and Their Interaction

Roots are not thought to have a rest period, and Harris (55) has shown that under favorable conditions root growth continues all year round, although the crowns of the plants enter a regular rest period. As against this, Bahgat (1) showed that x-ray treatment of roots produced chemical changes in them similar to those produced in their shoots when the rest of the latter was broken by means of x-ray or other treatments. However, these changes could also be produced in the shoots when the roots only were treated which could be interpreted as a remote effect of the stimulus but could also be due to a movement of enzymes or other substances from the roots, where they were liberated, to the tops. These chemical changes cannot, however, be exclusively related to the breaking of rest.

It is generally agreed that the individual bud is the seat of rest and that response to a stimulus is localized in the bud. (When it sprouts, it is supplied by the nondormant roots and stem.) Among the many experiments supporting the theory may be cited that of Denny & Stanton (33) who broke the rest of one of a pair of lilac buds by means of ethylene chlorhydrin; the closely adjoining bud remained dormant. Similar local stimulation can be exercised by different means, e.g., by immersing the bud in a waterbath at 30–35° C. for 9 to 12 hr. according to the method of Molisch. But when Krasnosselskaya & Richter (72) extended this period to 17 to 24 hr. the stimulus was transmitted to adjacent buds; the larger the treated portion of the stem, the greater the distance of action. Since, furthermore, the transmission of this stimulus was not interrupted by the removal of a ring of bark and since the stimulus moved in both directions, it was concluded that a substance was produced during treatment which, if transmitted in sufficient quantity, would move through the xylem into other buds.

Molisch's method has also been modified by raising the temperature of the bath. Vegis (135) who found even 35° C. not sufficient to break the rest of winter buds of *Hydrocharis* succeeded when he raised the temperature to 40° and 55°C. Although at 30–35°C., 12 hr. did not suffice to break the rest, only 15 sec. were required at the highest temperature.

Vegis' findings aroused controversy concerning the nature of the action of the warm bath. Earlier work of Boresch (12) had shown that the effectiveness of the treatment depended on a combination of raised temperature and anaerobic conditions, and that products of anaerobiosis were the active agents in breaking rest. The more recent findings of Krasnosselskaya & Richter (72), concerning the transfer of stimulus from treated to untreated buds, do not contradict Boresch's theory, as their findings involved only a quantitative and not a qualitative *novum*. On the other hand, the effectiveness of Vegis' treatment after only 15 sec. can hardly be explained by the synthesis of a product of anaerobic respiration. This was clinched by Vegis' finding of a Q_{10} as high as 58 which excludes the possibility of a primary enzymatic or chemical reaction. He suggests that some other process, such as denaturation of proteins or the separation of a lipid-protein complex, might be involved. In the writer's opinion, however, results obtained at the high temperature at which leaf primordia were injured, do not contradict the possibility that at biological temperatures, a respiratory mechanism is involved.

Returning to the question of localization of rest in the bud, an observation of Chandler (20) should be mentioned. Though the rest of an individual bud may be broken, the bud seems to grow better on a branch that has been subjected to a sufficient amount of chilling. As has been noted above, cambium cells apparently enter a resting condition (101). The question arises whether the cambium starts independently, to grow again, under the direct effect of chilling for example, or whether the cambium responds to a stimulus originating in the buds. Even if the latter is the case, Chandler's observation can be explained on the basis that if the stimulus comes from a single bud whose rest is broken, comparatively fewer cambium cells are awakened than if the rest of many buds is broken. The number of xylem vessels laid down is in proportion to the number of cambium cells which are activated; this, in turn, affects the supply of nutrients.

While the connection between the resumption of cell division in the cambium and the awakening of buds was already known, it remained for Soeding (117) to show that swelling buds provide a sudden, abundant source of auxin. He correlated the movement of this auxin with the basipetal resumption of cambial growth. Thus, the mechanism by which the rest of cambium cells is broken seemed quite clear, particularly since it had previously been shown that growth substances, applied to decapitated or ringed stems, activate cambial activity below. At this point, Gouwentak (49) showed that auxin alone was not sufficient to break the rest of cambial cells, but only supplied the required growth factor for cells whose rest had previously been broken or reduced by such means as ethylene chlorhydrin. This recalls the fact that, in previous experiments demonstrating the effectiveness of auxin, wounds were made in order to apply the auxin. However, the wounds themselves, by breaking the rest, may have conditioned the cells for growth. Thus, cold weather may act directly to break the rest of tree-bark, although the auxin required for its growth comes later from the buds. This transitional stage between breaking of rest and establishment of auxin supply might be worth in-

vestigating, particularly since favorable temperatures during this stage seem to bring about changes in the cell which make it more subject to frost-damage (38).

Gouwentak's findings do not, however, preclude the possibility that both the breaking of rest and the growth of cambium cells may be affected by substances derived from swelling buds. Thus, Krasnosselskaya & Richter (72), by inserting a bud of the same species which had been forced previously, were able to break the rest of buds of *Populus* and *Fraxinus* above and below that point. They went further (103); they introduced under the bark of resting lime and ash branches two to three drops of an aqueous pulp from warm-bath treated buds. About a week later the buds along the entire shoot showed clear signs of swelling, thus indicating that both the substances required for breaking rest and those required for initiating growth of distant buds, were supplied by the pulp of swelling buds; this would not have been the case if the warm-bath treated buds had been permitted to continue to grow *in situ*. Therefore, one must conclude that the process of grinding the bud tissues either liberated a substance or substances from the protoplast, or caused them to be produced when the bud's structure was destroyed. Though the wound-effect is one of the oldest known means of breaking rest, little work has been done in recent years on the elucidation of its mechanism.

In a recent paper, Reinders-Gouwentak (102) showed that in the activation of the cambium tissues in poplars, the female flowers were effective for a greater distance than were the male catkins. This may be due to differences in auxin concentration in male and female flowers rather than to a specific sex hormone, particularly, since vegetative buds have an even stronger effect than blossom buds. In this connection it might be recalled that Zollikofer (147) found that solutions of oestrone awaken the buds of *Aesculus* and other dormant branches standing in it. She also confirmed the observation that thyroxin has a similar action.

Certain Processes Associated With Rest

Although certain processes of the rest phenomenon have already been discussed, it seems desirable to deal with some of the mechanisms in greater detail.

Chemical changes.—There is a considerable literature concerning the chemical changes which take place during rest and at its termination. Formerly, the generally accepted theory held that the accumulation, during the growing season, of photosynthetic products such as sugars, gradually inhibited hydrolytic enzymes, thus stopping growth (62). This accumulation, it was believed, was slowly removed by respiration during the rest period, so that enzyme action could start anew. This theory was refuted by the work of Gardner (45). He found, in pear shoots, neither inactivation of enzymes in fall, nor reactivation in spring. He found, instead, the relative concentration of the components involved quite contrary to that required by the theory, inasmuch as the concentration of hexoses and sucrose increased during the rest period at the expense of starch. He found, also, a considerable ac-

cumulation of organic acids, but no changes in total or amide-nitrogen, fat, or soluble salts. Bahgat (1), however, noted also a hydrolysis of fats and an accumulation of fatty acids. The situation is somewhat different with the yellow poplar. According to McDermott (85), both starch and soluble carbohydrates decrease during rest, with a parallel increase in polysaccharides other than starch. After mid January when rest is probably broken, this trend is reversed. During this period, furthermore, the insoluble nitrogen decreases with a corresponding increase in the soluble nitrogen. Sell & Johnston (113) found that in tung terminal buds in late winter, rest already, perhaps, having been broken, a rapid decrease of sugars was followed by a decrease of polysaccharides and fatty constitutents disappeared.

Sell's work had the great merit that it was carried out on the buds and not on the entire shoot, as was most previous work, but the state of dormancy was not adequately defined. Thus, it is impossible to differentiate between the changes directly connected with the cessation of rest and those resulting from initiation of growth within the closed bud. We are indebted to Miller, Guthrie & Denny (88) for having bridged this gap in our knowledge concerning the sequence of processes during rest-breaking. Working with potato tubers, whose rest had been terminated with ethylene chlorhydrin, these workers recorded the changes in them at short intervals after treatment. They found an immediate increase in CO_2 output and a simultaneous decrease in citric acid and hydrogen ion concentration, followed by increased catalase and peroxidase activity and, later, an accumulation of sucrose and glutathione. This initial reactivation of the four-carbon-acid respiration seems to be of prime importance for the resumption of growth. In this connection, Bennett's experiments [as cited by Bahgat (1)] in breaking the rest of pear shoots by injecting them with citric, malic, or succinic acid, are of particular interest. The four-carbon acids may be considered (32) to provide the initial energy leading to the oxidation of sugars and to the conversion of amino acids into higher compounds.

Thimann (125) has shown that the four-carbon acids may serve another function, that of protecting the sulfhydril group as well as other groups containing the reduced sulfur which is essential for cell division. Guthrie found (e.g., 52) that a number of rest-breaking chemicals such as ethylene chlorhydrin, ethyl alcohol, acetaldehyde, and others stimulate the synthesis of glutathione which is one of the most important compounds containing the sulfhydril group. The rest-breaking property of these chemicals may be due to the synthesis of glutathione, which itself was shown to be effective in breaking the rest of buds on pear and apple shoots, among others. Miller, Guthrie & Denny (88) found the appearance of glutathione to be accompanied by a disappearance of sulphate which they consider to be the source of the sulfur for the glutathione molecule. The peptide for this synthesis might possibly be furnished by protein hydrolysis, as indicated by the appearance of catalase and proteinase during rest-breaking, observed by Toshevikova (128). While the two rest-breaking agents, thiocyanate and thiourea, do not cause the synthesis of glutathione, they do contain bivalent sulfur. Thornton (127)

points out that ethylene chlorhydrin and thiourea are strong antioxidants; he attributes their effectiveness in rest-breaking to anaerobiosis. Still, we must not be concerned with one mechanism only, because there may be several steps involved, as is indicated by the synergism observed between several rest-breaking agents used by Denny (31).

Anaerobic respiratory products have already been mentioned above as rest-breaking agents which are formed during warm-bath treatment. Bahgat (1) has shown that excluding oxygen from pear shoots effectively terminated rest. Furthermore, he ascertained the presence of acetaldehyde and ethyl alcohol in the treated branches, and demonstrated that either compound, particularly acetaldehyde, awakened the buds on pear shoots. Whether products of anaerobiosis act on rest in the course of intramolecular respiration through some mechanism other than glutathione has not, as yet, been determined. It should be added in this context that cyanide, which causes the accumulation of products of intramolecular respiration, is a strong rest-breaking agent (137). Even at a concentration too low to influence respiration, it inhibits resynthesis of the breakdown products of carbohydrate respiration [the Pasteur-Meyerhof reaction (17)].

It is of interest to note, too, that dinitrophenol which inhibits adenosine-triphosphate synthesis (81), and thus interferes with an essential factor in carbohydrate respiration, is also a rest-breaking agent. Weinberger (138) and Guthrie (53) tested a large number of phenolic compounds, several of which were shown to break the rest period of peach buds. Chandler *et al.* (22) showed the rest-breaking effect of dinitro-cyclohexylphenol on a large number of trees and shrubs, and Samish (106, 110) studied the rest-breaking effect of dinitrocresol on apples and plums.

The mechanism of action of dinitrocresol is likely to be similar to that of dinitrophenol. Loomis & Lipmann (81) point out that dinitrophenol, by depriving the cell of the energy of the phosphorus bonds, leads either to intramolecular or fat respiration, thus sparing sugars. At high concentrations it inhibits respiration. At low concentrations, however, Bonner (9) showed that it even increases rate of respiration, but because of the uncoupling of oxidation from phosphorylation, it inhibits growth and differentiation. Indeed, Oberle *et al.* (95) confirmed previous observations that dinitrocresol-mineral oil sprays increased the respiration rate of dormant buds of deciduous trees.

Thus, we find that aerobic respiration, stimulated by four-carbon acids on the one hand and anaerobic respiratory products on the other, lead to the breaking of rest. These facts have not as yet been reconciled in a unifying theory. This matter is complicated further by the results of Curtis (28) who, by displacing air with water in privet twigs, induced dormancy in the buds. Crocker (27) points out that in view of the fact that there exists no direct relation between the effect of a number of chemicals on respiration and on rest, there may be no fundamental causal connection between the two. Ruge (105) however, believes that two processes are required in order to terminate rest in seeds: first, stimulation of respiration to supply the required amount of energy, and second, the supply of growth-inducing auxins. These

two processes would correspond to the processes in cambium, and they may occur, also, in buds.

The auxin-inhibitor mechanism.—With increasing recognition of the role of auxin and related substances in growth processes, attention was directed towards their role in dormancy and rest. The theory that polar inhibition of lateral buds was due to excessive auxin concentration [van Overbeek (132)], led to the thought that a similar mechanism was involved in rest. Indeed, Kassem (68) was able to extract a much larger amount of "total" auxin from pear shoots at the begining of rest than later on; toward completion of the rest period, auxin concentration continuously declined. Eggert (39) showed that rest was terminated when the auxin concentration in apple buds dropped below about 0.25 μg. Furthermore, it was found that certain rest-breaking agents, such as thiourea and ethylene chlorhydrin, counteracted the inhibiting effect of excess naphthalene acetic acid ester which had been applied to the bud (30) and destroyed the apical dominance (127) thought to be connected with high auxin concentration. These results led to the conclusion that excess auxin brought about dormancy and that its gradual disappearance removed the growth inhibition. This theory was questioned by Skoog (115) however, and was disproved by Chan-Thom (24) who showed that an auxin concentration would have to be above 10^{-3} M in order to inhibit growth in pear, an amount much greater than that found in these buds at any time.

As against these findings with "total" auxin, diffusion methods or less radical extraction of buds gave a very different picture with the exception of cambium scrapings where Kramer & Silberschmidt (71) have shown a similar trend during rest as the above, i.e., the decrease in auxin; still in the cambium there may very well be a different mechanism than in buds. Thus, we have shown above that for the complete breaking of cambium rest, it is essential that auxin be supplied from another source, such as distal buds, while buds themselves have to supply their own auxin requirements. Bennett & Skoog (4) could not find any diffusible heteroauxin in resting pear buds, nor could Malan (83) find ether soluble auxin during dormancy, even where rest was prolonged considerably in the hot house. On the other hand, Bennett & Skoog found that the auxin gradually increased in the cold room while rest was diminishing. This was confirmed by Kassem (68) who showed that dormant buds were low in "free" auxin, which increased rapidly as spring approached. Similarly, van Overbeek (133) could not find the cocoanut milk factor in dormant apple buds which he had shown to be contained in nonresting buds. Finally, Chan-Thom (24) recently showed that at the time of entry into rest in July, the auxin content of lateral pear buds gradually diminishes; this trend is reversed during rest-breaking chilling. She obtained considerable increase of respiration in pear buds, a typical consequence of breaking rest, by applying low concentrations (10^{-3} to 10^{-8} M) of indoleacetic acid, thus confirming similar results obtained by Bennett & Skoog (4), who also broke rest by injecting tryptophane, which is required for the synthesis of auxin. Samish (108) broke the rest of apple buds by means of zinc sulphate sprays, a matter of interest since Tsui (129) and Kessler (70)

have recently shown that the zinc ion is a catalyst in tryptophane synthesis. These findings seem to indicate that auxin is required for the breaking of rest. On the other hand, they do not exclude the possiblity that the cell is previously conditioned by some other process, as has been shown for the cambium. These findings, moreover, do not explain what becomes of the auxin when rest is initiated, or what makes it reappear.

Hemberg (57) has put forward an explanation, derived from his work on the potato and on *Fraxinus* buds. In both the aqueous and the ether extracts of peelings from dormant potatoes, and in the scales as well as the inside tissues of *Fraxinus* buds, he found neutral and acid growth inhibitors (58, 59). The acid growth-inhibiting substance or substances were destroyed (60) when the tuber came out of rest naturally, or when the rest period was terminated by means of wounding, drought, cold, or ethylene chlorhydrin. While he has thus shown the connection between inhibitors and rest, he also established that the auxin content does not change either during rest or when rest is broken. A similar situation was found by Pollock (99) in maple buds, where the inhibitor system was shown to counteract the cocoanut milk factor, as pointed out by Steward (120). Recently, Spiegel (119) found two heat-stable inhibitors in the neutral fraction of the ether extract of grape cuttings. While the auxin did not show appreciable fluctuations in the course of the winter, the inhibitor concentration reached its maximum in January and fell to zero about two weeks before bud swell, at which time auxin showed a sharp rise. This late rise may have been due to initial bud development subsequent to the breaking of rest. In varieties with a lower chilling requirement there was an earlier disappearance of the inhibitors; these also were destroyed when the rest was broken by a period of chilling.

The finding of these inhibitor systems does not, in the view of the writer, contradict the theories of Bennett's school, based on auxin activity, but rather, indeed, explains them. Inasmuch as the auxin determinations had been made by the coleoptile method, the coleoptile curvatures resulting from the extract applied were due to the differences in response to auxin and to inhibitors. Thus, while the amount of auxin may have remained rather stationary, the rapid accumulation of inhibitors at the beginning, and the gradual destruction towards the end of rest, would result in the initial disappearance and final reappearance of the auxin action. This explanation was foreshadowed by the suggestion of Bennett & Skoog that during the rest period auxin was held in the form of a precursor or in some other inactive state. The differing curves for total, as compared with diffusible auxin, may have been due to the partial or total destruction of the inhibitors during extraction. The explanation put forth above need not exclude the possibility of specific differences in either auxin or inhibitor behavior. Kramer & Silberschmidt (71), for instance, reported that *Salix* under tropical conditions retained a high auxin level during winter; in *Populus* it was very much reduced.

A positive correlation between negative curvature of the *Avena* coleoptile (as an indicator of auxin less the inhibitor action) and rate of respiration was obtained by Chan-Thom and Spiegel. That the relative effect of auxin

and inhibitors may influence not only the rate, but also the nature of respiration, is indicated by the work of Christiansen & Thimann (26), who, though in a specific case, seem to find that certain inhibitors raise the respiratory quotient (R. Q.), while indoleacetic acid decreases it. If we assume that entry into rest is attended with an increasing dominance of the inhibitor over auxin, which is reversed during the ending of rest, then the measurements of Pollock (99) on maple buds showing a rise of R. Q. at the beginning of rest and its falling again with beginning of bud swell, would fit in well with the above mechanism. Pollock himself, however, explains his data on the basis that the entrance of oxygen is interfered with, and that this effect is modified by temperature changes. He found that at higher temperatures, a higher partial-pressure of oxygen is required in order to attain a certain R. Q. level, while a lower partial-pressure will be necessary at lower temperatures. Thus, the same limiting partial-pressure of oxygen, which produces a high R. Q. in summer, favors inhibitor formation and brings about rest. Low temperature in winter produces a lower R. Q. associated with aerobic respiration, which favors the oxidation of unsaturated inhibitors and thereby breaks rest. Even if a poor oxygen supply, at the time of entering rest, does not cause the formation of inhibitors, it may prevent the oxidation of such substances derived from the leaves (50) and thus cause the accumulation of inhibitors. Indeed, we find, in an early paper by Curtis (28), that he induced dormancy in buds by creating anaerobic conditions.

Whatever the mechanism involved, the situation seems to be different in pear buds, inasmuch as both Chan-Thom (24) and previously Bahgat (1), obtained trends in the R. Q. of pear buds, contrary to those of Pollock (99). They found a low R. Q. during rest, and unity before and after the rest period. Whether this is to be accounted for by fat respiration during rest in consequence of a certain enzyme-hormone constellation, or whether, and to what extent, the R. Q. in either case is affected by rates of gas diffusion, remains to be elucidated.

Physical factors.—While the bud scales may, at least with some species present a physical hindrance to the penetration of oxygen, diffusion either of gases or of solutes (and particularly of large molecules) may be affected by the physical state of the protoplast itself or its membranes. Indeed, early investigators postulated the existence of some such wall which, during rest, prevented contact between two partners of a reacting system, e.g., enzyme and substrate. Upon breaking down of the physical hindrance, metabolism can again be resumed, providing the energy for new growth. Bünning (17) summarizes a related view based on the observation that in summer, when buds enter rest, the plasma becomes more hydrated and gel-like. This, in his view, brings about rest since water, under such circumstances, is held more tenaciously thus making biochemical reactions more difficult. Bahgat (1) has shown that with entry into rest, the percentage of bound water increases; this bound water is released slowly in the course of the rest period, but rapidly, when rest is broken artificially. The removal of free water, with entry into the rest period, tends to reverse the activity of hydrolases to the synthesis of less soluble compounds, as is well known from the studies on the

mechanism of stomatal guard cells. Furthermore, according to the concept of Dixon (37), such removal of water affects total enzyme activity. He assumes the enzymes to be located in the water phase of an emulsion which is formed in parts of the protoplast. Amount and continuity of this water phase would then determine opportunities for the enzyme to meet a suitable substrate, which would affect the rate of metabolism. It has been found repeatedly—recently again, by Genkelj & Oknina (48)—that resting plasma shows a high viscosity which is decreased by severe cooling (47) such as would bring about termination of rest. Bünning also connects the action of the warm bath with colloidal changes. This may be related to the findings of Spiegel & Spiegel-Adolf (118) that anaerobiosis increases permeability of the cytoplasma. Guthrie (51) reports that rest-breaking ethylene chlorhydrin treatment increased the rate of leaching, permeability, and the conductivity of the protoplast. Such change in colloidal state associated with termination of rest may affect, first of all, such unstable complexes as the one which Sukhornkov & Bolshakova (123) assume to be formed between protein and hormone. The transition from gel to sol-state is thought to free the auxin from the complex. While the existence of such a complex does not seem to be sufficiently substantiated by experiment, much work has been done on the reverse effect—the influence of growth substances upon the viscosity and permeability of the protoplast.

Thus, Northen (94) indicated that auxin causes the less active gels to dissociate into liquefied colloids. This change in state frees the sulfhydril groups which then become active in metabolism. Pohl (98) has shown that auxin increases the water permeability of the protoplasm, which, in turn, leads to more rapid uptake of water. A recent paper on this subject, by Guttenburg & Beythien (54), emphasizes the speed with which permeability of the protoplast increases under the influences of growth substances; this, these investigators interpret to indicate that heteroauxin primarily affects the protoplast as a whole, rather than its outer membrane. This latter possibility has been demonstrated by Havinga & Veldstra (56, 136) who used a model of lipoid monolayers to show that under the conditions of their experiment, growth substances "opened" the membrane, while an inhibiting substance, such as coumarin, contracted it. The results obtained, however, have not so far, been correlated with physiological activity. Both the effect of auxin on the mass of the protoplast, as well as such "opening" of the cytoplasmic membrane are of particular interest in elucidating the mechanism of rest. Early investigators had postulated some such mechanism, and van Overbeek (134) had correlated the physiological impact of a series of petroleum oils on the leaf, with their ability to solubilize plasmic membranes. Samish (111) further showed that the activity of van Overbeek's series corresponds with the effectiveness of various petroleum oil sprays in breaking rest of apple buds. If the effect of various substances on protoplasmic membranes, e.g., that found by Havinga & Veldstra, could be firmly linked with the effect of these substances on rest, it would be an important step towards understanding the mechanism of rest.

In addition to the chemical and colloidal-chemical reactions which seem

to be active in the mechanism of the rest period, a photochemical reaction seems to be involved, at least with some plants. Thus, Niethammer (93) injected photochemical sensitizers into resting trees. In light, the sensitizers caused the breaking of rest, but did not affect rest in the dark. That light may, in certain cases, counteract the inhibition of seed germination by coumarin has been shown by Evenari (42). In view of the role of inhibitors in rest, this observation may provide a link between photoperiodism and rest. It has long been known that certain deciduous trees, when subjected to permanent illumination or long-day conditions, continue to grow without entering rest. As an exception Garner & Allard (46) showed, with apple, that entrance into rest was delayed if it was given a short-day period, while Bonner (10) observed that peach seedlings enter rest when held under short-day conditions. Van der Veen (131) reported similar results for several poplar species. On the other hand, with sugar maple, Olmsted (96) did not observe any correlation between photoperiod and either entrance into rest or end of rest in spring. Summer flush in ordinary daylight occurred only with 23 per cent of the seedlings; 72 per cent required a 20-hr. day; while none grew under a 9-hr. light period. The fact that the date of spring growth from a leafless branch was not influenced by daylight may be expected, since no leaves were present to receive the light stimulus. On the other hand, the fact that this species did not react to long days when entering rest, though other species do, shows that photoperiodism is not a factor universally involved in rest. It may be questioned whether growth stoppage before the second flush is true rest; but even here we find varietal differences in response to day-length. It would be interesting to know whether the seedlings with differing photoperiod had, also, different chilling requirements. Photoperiodism, then, seems to be a factor in rest, at least with some species and varieties. Correlations betweeen photoperiodic behavior of rest and other phenomena in the life of the tree may lead to conclusions of fundamental importance.

There exist certain parallel features in the photoperiod and rest period. Thus, while flowerbud differentiation frequently requires a different period of illumination than vegetative buds require for growth, flower buds, generally, will also have a different (shorter) chilling requirement than vegetative buds. Vegetative terminal buds have a lower chilling requirement than lateral buds (among others see 40). The apex is, in certain species, the exclusive location of blossoms; in others it is the preferred point of blossom-bud differentiation. Thus, while blossom-bud differentiation, frequently conditioned by the photoperiod, may be associated with a lower chilling requirement, many plants fail to flower at all unless exposed for a definite length of time to low temperature (8, 142, 144). Again, high temperatures repress flowering even to the extent of nullifying an initial thermo-induction (126). In photoperiodism, light nullifies the dark reaction. Lang & Melchers (76) found that leaves exposed to short-day conditions develop an inhibitor which is removed by long-day treatment. We have seen that an inhibitor, found by Pollock (99), is formed in maple in warm temperatures; similar substances in *Fraxinus* are, according to Hemberg (59), destroyed by cold. This reversibility

of the effect of temperature on rest was indicated by the finding of Bennett (3) who showed that the chilling requirement of pears was increased when cold periods were alternated with warm periods. Vegis (135) reports for *Stratoides* buds that 1½ to 6 hr. daily exposure at 25° C. nullifies the effect of a cold period at 5 to 15° C. during the rest of the day. Such parallelism may indicate that perhaps some of the mechanisms involved in both physiological phenomena are similar. It may be that the unsaturated lactones, known to be photosensitive and to be involved in such growth processes as rest, will prove to be one of the links between photoperiodism and rest. Borthwick *et al.* (13) call attention to the fact that two pigments and two reactants are involved in photoperiodism, all four of which might be expected to vary in plants. On the basis of the many possibilities thus created, they ask "will here be found part of the explanation for dormancy of buds, biannual character, vernalization, ripeness to flower, and many other phenomena in plant growth?"

APPLIED PHYSIOLOGY

Horticulturists have always been interested in the rest period, particularly in connection with the forcing of hothouse plants. It is only rather recently, however, that Weldon (141) recognized that unfulfilled chilling requirement, causing prolonged dormancy, may represent a serious problem under outdoor orchard conditions. Since that time, prolonged dormancy, also called delayed foliation, has been recognized in most warm countries, as far north as the northern coast of the Black Sea (104) and as far south as South Australia and the Buenos Aires region (77). In those areas, after a warm winter, chilling requirements greatly affect the date of bud opening. Species or varieties with a lesser chilling requirement will open earlier. In addition, even on the same tree the flower buds, which have a lower chilling requirement, will open much earlier than the vegetative buds; among the latter, the terminal buds open sooner than the lateral buds. This precedence of the terminal buds intensifies polar inhibition, and, therefore, many of the lateral buds will remain closed. Since, in addition, both blossom and vegetative buds on the least vigorous branches open soonest (23), it is clear that prolonged dormancy results in a prolonged period of bloom and foliation. We have, therefore, to distinguish two phenomena: the general delay of bud opening, and its irregularity. Each, of course, causes certain horticultural difficulties which have been the point of departure for much research. Attempts to control both of these aspects of prolonged dormancy met the difficulty that, as has been pointed out, at any one date the different buds are in different stages of development with respect to rest. Thus, buds still in midrest will not be affected by any treatment safe under commercial conditions. Therefore any rest-breaking spray, applied at a time early in spring when there are still numerous buds in this early stage, will affect only a small proportion of the buds, which, of course, will open very early (107). While in this way delayed opening is prevented for part of the buds, irregularity of bloom is accentuated and may even lead to two separate periods of bloom

and foliation on the same tree. Furthermore, it should be pointed out that if the spray is applied very early in the season, e.g., in January, damage may result, inasmuch as fewer buds will open on such trees than on the untreated controls (110). If, however, the treatment is applied at an advanced stage, when most or all of the buds are already in late rest and therefore susceptible to the stimulating action, uniformity of bud opening will be attained at the sacrifice of earliness. Abscission of the buds or parts thereof, which is a corollary of prolonged rest, is not affected by such spray treatments.

Three groups of material have been used as forcing sprays. Various salts were found to be forcing agents, but early observations have not been followed up. Oil sprays have been found useful with species or varieties having lighter rest. Formerly, oils of plant and animal origin were used, but lately these have been replaced by mineral oils, which were introduced into orchard practice by Black (5) for the purpose of breaking prolonged rest. They have a two-fold effect. First, the oil coats the dormant branch (35) and thus is likely to bring about anaerobic conditions, the products of which would be active in breaking rest. Oberle *et al.* (95) have shown that, in addition, oil penetrates the buds and even the intracellular spaces of the rudimentary leaves. Samish (111) has shown that even inert pure paraffin oil has some forcing action. Secondly, it has also been shown that the effectiveness is increased with increase of unsaturation, i.e., decrease of U.M.R. (unsulfonatable residue) of the oil. Space does not permit discussion as to whether all these unsaturated compounds or part of them are effective in breaking rest. While, thus, in mild cases of prolonged rest, sprays of medium heavy mineral oils with relatively low U.M.R. may be effective in normalizing the tree and have found their use with prunes in California (65), it is necessary, under more severe conditions, to fortify the oil with, e.g., phenolic substances, whose rest-breaking properties have been discussed above in connection with respiration.

In the third group of spray materials, the phenolic substance, dinitro-cresol has been found most useful in Israel (106) and South Africa (67), greatly increasing the effect of mineral oil sprays. Lately, Micklem & Jeffrey, cited by Black (6), have also used it in aqueous solutions on peach trees, which are sensitive to mineral oil.

In order both to predict the necessity for treatment and to time it correctly, it became desirable to correlate winter climate with prolonged rest phenomena. In addition, the climatic requirements of different species and varieties, with respect to rest, had to be established. This involves at least two variables: evaluation of climatic conditions, such as temperature, in terms of degrees and length of period; and the determination of the stage in the development of the plant in which the climatic stimulus is effective. Weldon (141) observed the length of shade-period which, by lowering bud temperature, was effective in preventing prolonged rest, and found that the critical period extended over the two central winter months. This was verified by de Villiers (36). On theoretical grounds, however, the question arises whether a cold period during after-rest, e.g., a cold spell in spring, might not be more effective than the same cold period during mid-rest in winter.

The degree of cold required for breaking rest seems to be limited by a threshold value below which any temperature will have a similar effect with respect to breaking rest, while, obviously, very low temperatures may also retard development during this period (73). Weldon found, for peaches, that this threshold temperature lies between 9.6°C. and 9.8°C. average mean temperature for the two coldest winter months, while Hutchins [cited by Yarnell (146)] concluded that 7°C. was the threshold chilling temperature for peach buds. But these results are being applied uncritically for other species which seems hardly permissible. Indeed, Flemion (43) found that for post-maturation cold treatment of embryos (which may very well be related to chilling requirement of buds) the optimum temperature differs from 1–10°C. among different species. De Villiers (36) suggests, on the basis of his observations, that the threshold temperatures for chilling of different fruit species may lie below 9°C. for apple buds and as high as 12°C. for almond. Exact determinations are not as yet available. As these studies have been made on the opening of buds, they do not apply to bud drop. Recent experiments of Black (6) would seem to indicate that bud drop may be due to exposure, during the rest period, to even short periods of relatively high temperature.

The chilling requirement, i.e., the number of hours at or below 7°C. required to terminate rest has been determined for a large number of varieties (29, 40, 82, 139, 146). Frequently, greater differences in chilling requirement have been found among varieties of the same species than among various species. Thus, among blueberry varieties, according to Darrow (29), the chilling requirement varies from 260 to over 1000 hr. Yarnell (146) measured only 200 hr. for the Saucer peach, while Weinberger (139) recorded 1150 hr. for Mayflower blossom buds; and while Vinifera grapes are known to have a relatively low chilling requirement, the Concord grape has been shown to require as many as 3500 chilling hr. While such measurements provide useful information concerning the relative adaptability of different varieties for warm countries, climatic analyses permitting any forecast of time of blossoming, etc., meet with numerous difficulties. As has been pointed out, the threshold temperature for different species is not known. Furthermore, air temperature itself is not always related to effective temperature in the bud, since, as de Villiers (34) has shown, the radiation temperature may be quite high. Finally, measurement of the chilling temperature alone will not suffice under orchard conditions for we have seen that high temperature may reverse the chilling process to an extent which is not known. Determination of these factors under controlled conditions seems to be required, in order to place climate-rest correlations on a firmer basis.

Before closing this section, mention should be made of another effort of horticultural research, i.e., the prolongation of dormancy when spring frosts are likely to kill early bloom. The main approach used is that of creating an inhibition by an excess of growth substances and recently, also, by means of maleic hydrazide. Winkelpeck (145), using a spray of 125 p.p.m. naphthalene acetic acid and related substances, obtained an 11 days' delay of bloom with

peaches, but had poorer results with plums and cherries. Mitchell & Cullinan (89) had less success with blossom opening than with sprouting of vegetative buds. Marth *et al.* (84) found sodium and potassium naphthaleneacetate to be most effective when applied between August and October, but obtained delay only at injurious concentrations. This was confirmed by Tukey & Hamner (130) who associated severity of injury and degree of retardation of blossoming, using a mixture containing naphthaleneacetic acid and 2,4-dichlorophenoxyacetic acid. The maximum delay of midbloom was only two days, but it may very well be that since not all buds responded, the date of midbloom did not give a correct picture, and those buds which were sufficiently delayed might have saved the crop. At any rate, this treatment is not as yet satisfactory for commercial use, a conclusion which was also reached by Sell *et al.* (114) for tung buds.

Additional experiments in this direction were performed with maleic hydrazide, an auxin inhibitor which acts by competition [Leopold & Klein (78)]. Inhibition of polarity, retardation of growth, and in some cases [such as with raspberries, Kennard *et al.* (69)], delay of bloom was obtained. This frequently gives the impression of prolongation of rest, particularly with tubers and bulbs, but it is not as yet established that maleic hydrazide directly prevents the breaking of rest and thereby delays flowering.

Some General Considerations

The great differences encountered among species and among varieties as to chilling requirement, raised the question as to what caused these differences and what caused the cyclic recurrence of rest even where climatic conditions were favorable to growth. Mueller-Thurgau (91) and Howard (62) summarized the attitude of their time, which was governed by the natural philosophy of Haeckel. They supposed that growth inhibition due to cold, drought, etc., was fixed in the course of time in the plasma and became fixed as a hereditary rest period, a hypothesis very reminiscent of the Lysenko theory. As against this, Chandler (20) pointed out that species from cold countries, e.g., *Malus baccata*, do not necessarily have a long chilling requirement. We may, rather, find a mechanism involving mutation, such as was shown in the case of an apricot sport described by Lammerts (74) in which short rest proved to be a dominant factor in breeding. Bonner & Galston (11) mention that the short rest factor in peaches is recessive. Lesley (79) has shown that chilling requirement depends on multiple genes, some of which are intermediate in character. We can very well suppose that types arising in this way would then be subjected to the survival of the fittest. In this connection Chandler called attention to the fact that in cold regions plants with a long chilling requirement would not necessarily be at an advantage, since at the prevailing low temperatures even plants with a short chilling requirement would remain dormant after rest was broken. On the other hand, plants with a short chilling requirement would be damaged in countries with warm winters and occasional winter frosts. It might be added that in regions whose mild winters approach tropical conditions, only plants

with low chilling requirements would tend to survive or would be selected or bred by men, as is being done at present on a large scale (6, 74, 79, 140).

This Darwinian hypothesis may not, however, satisfactorily explain the universality of cyclic growth, of which the rest period is only a special case. Smolin (116) links dormancy with a theory which assumes that the potential of life activity gradually decreases, during cycles of aging and rejuvenation. Unfavorable growth conditions cause aging for which dormancy provides the rejuvenating partial recuperation. It is to be hoped that physiological and biochemical research will provide the necessary substantial basis for this hypothesis—or will furnish a new one.

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POSTHARVEST PHYSIOLOGY OF FRUITS AND VEGETABLES[1]

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The purpose of this review of postharvest physiology of fruits and vegetables is to evaluate pertinent findings published since Biale's (3) review in 1950 with particular emphasis on the role of volatile emanations in certain physiological diseases, notably apple scald. For the sake of convenience the subject matter is discussed under the following headings: (*a*) functional diseases related to volatile emanations, (*b*) volatile emanations and respiration, (*c*) chilling injury, and (*d*) the effect of growth substances on postharvest behavior. No attempt will be made to review the great amount of literature on changes in composition that take place in fruits and vegetables after harvest. Research on carbohydrate transformation in crops such as green peas, sweet corn, potatoes, carrots, bananas, apples, and pears, and on pectin and acid changes in fruits and vegetables has been covered in reviews by Smock (86) on deciduous fruits; Magness (55), Hawkins (33), Brooks (6), and Wright (101) on fruits and vegetables; and Miller (61) on citrus fruits. Changes in vitamin content of fruits and vegetables after harvest, particularly vitamin C and provitamin A, have been studied but with certain exceptions the results are of more interest to the nutritionist than to the plant physiologist.

FUNCTIONAL DISEASES RELATED TO VOLATILE EMANATIONS

Postharvest functional diseases of fruits and vegetables have received the attention of many workers. A description of these disorders and a review of what is known about their cause would be out of place here and might well be the subject of a separate review. Some of these diseases are essentially manifestations of chilling injury and are mentioned under that heading. Soft scald, internal browning, or brown core of apples, breakdown or browning of the flesh of peaches, plums, avocados, pineapples, melons, tomatoes, peppers, and eggplant, and skin pitting of citrus fruits, cucumbers, summer squash, and several other fruits and vegetables are examples of abnormalities arising from exposure to low temperatures. Other functional diseases are thought to be signs of senescence. Certain types of internal breakdown of apples and pears that appear in over-mature fruit or fruit held too long in storage fall into this classification. Other physiological diseases are known to

[1] The survey of the literature pertaining to this review was concluded in November, 1953.

be caused by lack of oxygen. Black heart of potatoes and brown heart of apples are two well-known disorders of this type.

A number of physiological diseases of importance as storage and market diseases of fruits and vegetables are essentially not postharvest for they often appear in the orchard or field. Water core of apples, related to high temperatures late in the season and delayed picking, is one of these diseases. Cork spot of apples, stony pit of pears, and a certain type of internal breakdown of beets, all related to boron deficiency, are other examples. Bitter pit of apples thought to be caused by death of starch-filled cells by excessive transpiration is another functional disease appearing before harvest but it often becomes more severe in storage.

APPLE SCALD

Early studies to develop a control.—A disorder of apples, known as apple scald or superficial scald, deserves attention in this review if we are to cover adequately the effects of volatile emanations on fruit physiology. This disease has caused heavy losses in stored apples and still does in years when apples are very scald-susceptible. Scald is believed to be caused by the accumulation of apple volatiles in the apple skin but what these volatiles are and how they produce the injury is not clearly established.

As early as 1903 Powell & Fulton (76) concluded that the disorder was physiological in origin and was not caused by bacteria or fungi. The brown discoloration of the skin of the apple characteristic of the disease gave it the name "scald" for the skin had a brown, cooked appearance. Growers thought scald was due to overheating of the fruit, or to low storage temperature or to some orchard effect. Over 50 years ago, Powell and Fulton showed that fruit picked too early was particularly susceptible to scald, that long storage until March or April favored it, that more scald occurred at 36°F. than at 32° and that the temperature at which the fruit was held when removed from storage had a marked effect on scald development. Varieties of apples were found to differ in susceptibility and the most susceptible ones were listed. Among a number of treatments that Powell and Fulton tried on late-stored apples already showing some scald, olive oil, vaseline, and paraffin retarded scald development. Here we have the first indication that the causal agent might be absorbed by oils or waxes. Holding the fruit in nitrogen, or submerging it in water, prevented scald, and holding it in oxygen caused more rapid development. These workers concluded that scald was caused by a mixing of the cell contents on premature death of the cells and their browning by oxidation through the influence of the normal oxidizing ferments of the cell.

Brooks & Cooley (7), in a preliminary statement in 1916, reported that high humidities favored scald, for it developed in apples held in saturated atmospheres in closed but not sealed containers. Kidd & West (46) found, in 1932, that apples in gas storage at 38°F. developed less scald at 80 per cent humidity than at 96 to 98 per cent and that fruit wilting severely because of

scab infections also developed less scald than apples not suffering so much wilt. They concluded that the rate of water loss was one of the factors concerned in scald and that conditions favoring water loss would aid in removal of the volatiles responsible for scald. Additional research by Brooks & Cooley (8) indicated that humidity was not important and showed that lack of aeration was the essential factor in scald production. As storage temperature was raised from 32°F. scald developed sooner, about 1 month earlier for each 9° rise up to 59 or 68°F. At 86°F. no scald developed but breakdown of the flesh occurred. Fluctuating storage temperature had no effect on scald development and rate of warming upon removal from storage was unimportant. When immature fruit was held in a well-aired place before storage, the susceptibility to scald was reduced. This, in effect, was like picking the fruit riper. Brooks & Cooley (8) concluded that scald was produced by abnormal respiratory conditions, that the fruit must respire to produce scald, that sealing the fruit in tight containers prevented it but caused other abnormalities, and that scald increased with temperature as does respiration up to a certain limit, about 86°F. The inhibition of browning at 32° was believed to be due to the suppressing effect of low temperature on the oxidizing enzymes of the skin. Brooks and Cooley believed that the injury causing scald was cumulative and that apples did not retain the scald tendency if removed to more favorable conditions before a certain critical period.

Further work by Brooks, Cooley & Fisher (9) brought them to the conclusion that they must be dealing with a volatile or gaseous substance other than CO_2 produced by the metabolism of the apple as the causal factor for scald. Scald could be prevented by wrapping apples in paper wraps impregnated with olive oil, cocoa butter, vaseline, or beeswax, and it could also be prevented by air movement in storage at 11 to 22 ft./min. Intensity of air movement rather than renewal of air were the essential aspects of aeration in scald prevention. Thorough aeration during the first 8 weeks of storage was more beneficial than later aeration. Brooks, Cooley and Fisher also found that CO_2 concentrations of 1 to 6 per cent tended to prevent scald and that holding apples for 3 days at 86°F. or 6 days at 59° in atmospheres of CO_2 made susceptible apples immune. This was believed to be due to retardation of ripening induced by CO_2 and hence slowing down of respiration and volatile emanations. Brooks, Cooley and Fisher tried to prevent scald by holding the apples in oxygen or treating them with ozone believing they might in this way oxidize the harmful volatiles but they were not successful. Smock & Van Doren (90) many years later obtained some control with ozone by using much higher concentrations.

Brooks, Cooley & Fisher (9) were able to produce an injury similar to scald by exposing apples to vapors from 10 to 20 per cent solutions of ethyl acetate, amyl acetate, or ethyl butyrate, all in ethyl alcohol, whereas vapors from ethyl alcohol, acetic acid, a mixture of the two, or formic acid did not produce typical scald injury. A large number of substances were found to be capable of absorbing the apple volatiles involved in scald development.

The best ones were olive oil, mineral oil, apple wax, lard, tallow, butter, peanut oil, and castor oil incorporated in paper wraps. Animal and wood charcoal gave only fair to poor control. More intensive studies on the control of scald with activated carbon were to come many years later (26, 89). It was of interest that the good absorbers like olive oil controlled scald on apples several layers away from the wrapped fruit. Some other observations of interest were that apples which became greasy in storage from development of natural wax did not scald, that ventilated barrels reduced scald if the room was ventilated as well, and that heavy irrigation of orchards produced a forcing effect that made both large and small apples susceptible to scald. Brooks, Cooley & Fisher (9) concluded that the disease was due to the accumulation of esters or similar products of the apple in the tissues of the fruit and in the surrounding air and that these vapors can be carried away by air currents or absorbed by fats and oils.

The investigation on apple scald had reached the stage when commercial control with oiled wraps was close at hand. Large-scale tests were made in the apple-growing areas with paper impregnated with mineral oil and it was found by Brooks, Cooley & Fisher (10) in 1923 that the paper should contain not less than 15 per cent oil by weight. Applying oil or waxes directly to the apple reduced scald but not as much as the use of oiled paper. Smock (88) came to the same conclusion in recent work. Kidd & West (46) found that apples treated with a fine spray of mineral oil developed almost as much scald as untreated fruit and they concluded that oil applied to the surface of the apples may prevent the escape of the harmful volatiles through the lenticels or other breaks in the cuticular surface of the apple.

Critical period for development.—Further investigations on the critical period for scald control indicated that oiled wraps could still be used advantageously on some varieties of apples even after 8 or 12 weeks' storage. Brooks, Cooley & Fisher (10) recognized 4 stages in the development of apple scald. The first period started at picking date and extended for 6 to 8 weeks in storage.

Scald-producing agents were most active during this time and scald could be prevented by aeration or use of oiled wraps. The next 5 to 8 weeks comprised the second period and preventive measures were of little avail and the fruit was doomed to scald if left in storage long enough; but if removed from storage before the end of this period apples might not show scald even upon warming. The third period was the rest of the cold storage life. Then the fruit was potentially scalded, certain cells were practically dead, but the fruit remained green and appeared almost normal if not exposed to warm air. The fourth period was the life of the apples after removal from storage, when the skin turned brown and completion of the death processes took place.

Kidd & West (49) were prompted by these findings (10) to determine when the critical period for scald development occurred in English varieties of apples and concluded that the second 3-week period of storage was most critical for normally harvested fruit and that the third and fourth periods of 3 weeks were also critical for immature fruit. Intermittent warming of apples

every 2 weeks for 24 hours at 59°F. controlled scald and warming every 4 weeks gave marked reduction, lending further support to the belief that accumulation of volatile products at cold storage temperature was the cause of scald. Kidd and West found that scald susceptibility fell to zero in apples held at unrefrigerated temperatures until the climacteric rise of respiration was completed.

Volatile production in relation to scald.—Kidd & West (49) found that volatile production as judged by taste and smell of water through which emanations were bubbled did not begin until about 6 weeks in storage, reached a maximum in the following 7 to 8 weeks and was not related to the critical period for scald control.

Smock & Southwick (88) measured the nonethylenic volatile production of McIntosh apples harvested on different dates and held at 74°F. Volatile production and scald were somewhat higher for fruit harvested in 1941 than for 1943 fruit. Fidler (21) in contrast observed an inconsistency in evolution of odorous volatiles and scald production, for in a season of high volatile production he had less scald than in a season of low volatile emanation. Fidler (20) also found that a variety of apple, such as Cox's Orange Pippin, not susceptible to scald, produced more volatiles than Bramley Seedling, a scald-susceptible variety. Fidler (21) did not get scald control by lowering the odorous volatile content of the storage room air with activated carbon and obtained only fair control with brominated carbon, which lowered ethylene content as well as removed more nonethylenic volatiles than activated carbon. Oiled paper gave the best control of scald, yet it gave only a slight reduction in odorous volatile level in the storage chamber.

Smock & Southwick (89) reported good control of scald with activated carbon when apples of a single variety were stored in a room and as good control in those of mixed varieties where scald susceptibility is increased as was obtained by shredded oiled paper. Gerhardt, Sainsbury, & Siegelman (26) failed to get significant reduction of scald on either apples or pears by air purification with activated carbon or brominated carbon.

In an attempt to reconcile the apparent conflicting evidence for scald being due to a single factor Fidler (21) proposed a theory that scald may be caused by two factors, one produced early in the season by a substance not very volatile at cold-storage temperatures. This factor, called X, can be absorbed by oiled paper. The second factor, Y, produced later in the season, is fairly volatile and is capable of producing scald in combination with X, but if X is absorbed no scald appears. Fidler assumed that the bulk of the odorous volatiles, V, have nothing to do with scald production; hence their level and rate of production are no indication of scald potential. Activated carbon absorbs these volatiles and also part of Y, according to his theory, but since X is present in apples not protected with oiled wraps, scald develops. Brominated carbon absorbs the main bulk of the volatiles also and more of Y than unbrominated carbon, and hence gives better control of scald. Oiled wraps are evidently not very retentive of the odorous volatile, V, for

they did not reduce volatile levels appreciably in storage. They may absorb most of Y and some of X, removing these materials from the zone where they cause scald. The beneficial effect of warming apples in preventing scald, observed by Kidd & West (49), might be explained by allowing more Y to escape by increasing its volatility. The often-observed freedom from scald of the high-colored waxy side of the apple or of waxy varieties may mean that the wax acts as a natural oil wrapper. These are speculations of unproven merit.

Shutak, Christopher & Pratt (83) investigated the effect of treatments that would promote escape of volatiles from the skin of apples, such as removal of wax with pumice, alcohol, or brushing and found that when applied as prestorage treatments they greatly reduced scald and had some effect when applied as late as January. They demonstrated, as Powell & Fulton (76) did in 1903, that cool, post-storage temperatures reduced the extent and intensity of scald. They concluded that the waxy coating appeared to serve as a barrier or regulator controlling accumulation of scald-producing substances. They believed that most of the cells in scalded tissue are killed after removal from storage and threw doubt on the concept of early latent injury followed by oxidative browning upon removal to high temperature.

Identification of apple volatiles related to scald.—A considerable amount of work has been done on the chemical nature of apple volatiles. Power & Chestnut (77) in 1920 identified methyl, ethyl, and amyl esters of formic, acetic, caproic, and caprillic acid as odorous constituents of apples. Henze, Baker & Quackenbush (35) reviewed this field of work in connection with a study of volatiles adsorbed by activated carbon in apple storages. They concluded from their work and that of other investigators, notably Thompson (92), that saturated acids and alcohols with 1 through 6 carbon atoms can occur in the free and esterified forms in the volatile emanations of apples. The relative proportion of the different components may vary from sample to sample, reflecting varietal differences in aroma, flavor, and maturity. Possibly the amount and type of certain compounds might lead to explanation of varietal differences in storage-scald susceptibility. The fact that most of the volatiles were esters, whereas the volatiles of apple juice were found by White (98) to be mostly alcohols led Thompson (92) to suggest that esters may pass through the lipoid phase of the cuticle of the apple more easily than alcohols and thereby be preferentially evolved.

In a study of volatile products of apples in relation to scald, Huelin (37) found that Granny Smith apples held at 86°F. gave off volatile aldehydes and ketones as well as alcohols and traces of esters. Acetaldehyde was found to be the predominant aldehyde along with small amounts of propionaldehyde and acetone. He believed that acetaldehyde probably arose from the decarboxylation of pyruvic acid, an important intermediate of respiration, and that aldehydes were to be expected in the interconversion of acids and alcohols known to be present in apples. Acetone was probably a product of fatty acid metabolism.

Thompson & Huelin (93), turning their attention to the production of volatile esters by Granny Smith apples, found that increasing the rate of air flow past the apples gave an increase in ester production at 68°F. The amount produced upon removal of the apples from storage increased with storage life at 32°. The increase in volatile ester production with longer storage was correlated with increased alcohol production believed to be due to lowered efficiency of the oxidative system of the apple as part of the process of senescence. The course of respiration declined in fruit held at 68°, whereas the rate of ester production increased. Early-picked fruit gave off a smaller amount of volatile esters than fruit picked later even though early-picked fruit is more scald-susceptible than late-picked. This work provided additional evidence that scald was not directly related to the amount of volatiles the fruit give off [see Fidler (21)], but it does not follow that volatiles or precursors of volatiles are not in some way involved in the disorder. Thompson and Huelin reported that experiments with synthetic esters failed to provide evidence to support a direct relation between volatile esters and scald.

Griffiths & Potter (27) in tests on King Edward apples observed that a high concentration of volatiles in storage at 41°F. (5°C.) coincided with a marked increase in scald. They believed that it was unlikely that ethylene was the causal agent and believed that the causal agent was a compound of higher molecular weight. They obtained the most severe scald in apples held in the presence of their own volatiles. These apples were shown to have had their volatile production suppressed by the presence of volatiles as compared with those in similar storage from which the volatiles were removed. Griffiths and Potter suggested from this evidence that the causative conditions for scald may be the accumulation of precursors of the odorous volatiles rather than of the volatiles themselves. If this were true, conditions that would favor volatile production and evolution would help to prevent scald. This theory is tenable with the good effect of ventilation, holding at high temperatures, and other means of removing volatiles.

The work reported here on volatiles and the earlier work of Power & Chestnut (77) and Walls (96) provide evidence of the complexity of the problem of identifying the causal agent or agents of scald among the esters, acids, alcohols, aldehydes, and ketones present in apple volatiles. The question of the cause of scald still remains unsolved.

Skin composition and scald.—The natural coating of the apple skin has attracted attention for it is important in the physiological behavior of the fruit, forming a barrier to the diffusion of volatiles, water vapor, carbon dioxide, oxygen, nitrogen, and other gases. As indicated earlier, apple wax may be related to scald, for waxy apples are usually not susceptible. Apple skin has a coating consisting of waxes [Sando (80); Chibnall *et al.* (13); and Markley, Hendricks & Sando (58)], ursolic acid [Sando (80, 81)], and an oil [Gane (23)] made up mostly of unsaturated esters. In addition, the skin contains a material called cutin, which is water-repellant and contains lipoids [Markley & Sando (56)]. In later studies, Markley & Sando (57) found that wax, oil, ursolic acid, and cutin increased during maturation and storage at 32°F.

(0°C.). Huelin & Gallop (38) investigated further the natural coating of apples and the chemical and physical properties of the oil, wax, ursolic acid fraction, and cutin. These authors (39) found in studies of changes in storage that the oil fraction increased and reached a maximum 3 to 4 times its original concentration, that its increase was reduced by "gas" storage and that later pickings had a higher oil content than earlier ones. The iodine number of the oil increased as its concentration increased. Wax, ursolic acid, and cutin fractions increased less than oil during prolonged storage. The fatty esters of the oil fraction were produced most rapidly at the beginning of storage; subsequently, these nonvolatile esters declined while the rate of volatile ester production increased. There was no definite correlation between the oil content and the resistance of the skin to gaseous diffusion, although both increased during storage. The increase in oil during storage indicated a definite production of oily substances by the apple tissue, produced by the epidermal cells and secreted into the cuticle, or transported to the surface from other parts of the apple.

Evidence was obtained that apple cells produce fatty acids (mostly esterified) and other fatty substances. The apparent cessation of oil production may be due to (a) inactivation of the lipoid-producing systems, (b) exhaustion of lipoid precursors, or (c) the effect of increasing oil concentration on the lipoid metabolism. The first alternative was thought to be plausible as there is failure of other synthetic mechanisms during senescence. The fatty acids of the oil show a variation in chain length from 12 to 22 carbon atoms. The volatile esters produced by the Granny Smith variety of apples have been shown to be derived from lower acids and alcohols with a maximum chain length of 6 carbon atoms. The production of volatile esters can be linked with the accumulation of nonvolatile fatty acids in the oil fraction, but the peak of volatile ester production comes much later than that of the nonvolatile fatty acids.

Other work on the skin of apples in relation to scald is of interest. In the U. S. Department of Agriculture laboratories at Wenatchee, Washington, it has been shown that the phenolic content of the skin of apples decreases with scald development, indicating that the brown color is formed by the action of enzymes on phenolic compounds and in this respect resembles browning of peaches and other fruits. Respiration determinations on incipient-scalded skin and normal skin of apples were made as part of these studies. Volatiles from apple storage rooms did not increase the respiration of apple skin. Scalded skin had a lower respiration rate than normal skin. The reduction in respiration was quantitative, indicating that a portion of the cells of the skin are no longer functioning and are presumably dead.

Present status of knowledge.—It is apparent from this review that in cold storage some varieties of apples produce a substance which is toxic to the surface cells and ultimately causes death and oxidative browning of the tissue. This disorder, apple scald, can be prevented or minimized by allowing the fruit to become riper before storage or by accelerating the removal of

some volatile substance from the fruit. Wrapping apples in oiled paper or packing them in shredded oiled paper is the practical method used to prevent apple scald. Treatments that favor loss of volatiles from the fruit are helpful in controlling scald. Air movement past the fruit, warming the fruit intermittently, and making the skin of the apples more gas-permeable all are of benefit. Total volatile output of apples is not a measure of scald susceptibility. Nonsusceptible varieties may give off more volatiles than susceptible ones and early-picked fruit, most susceptible to scald, has a lower output of volatile esters than late-picked fruit. Some workers have suggested that the causative agent for scald may be precursors of odorous volatiles rather than the volatiles themselves and therefore conditions which favor volatile production and evolution help to prevent scald. These precursors or the volatiles themselves are still unidentified. Ethylene has been ruled out as the causative agent. Recent work throws doubt on apple esters, as a direct cause although in early work a scald-like injury was produced artificially by vapors from alcoholic solutions of a number of esters occuring in the volatiles from apples. Saturated acids and alcohols with 1 through 6 carbon atoms occur in the free and esterified forms in apple volatiles; also aldehydes, principally acetaldehyde, and ketones have been identified in apple volatiles. The volatile esters of one variety of apples were shown to be derived from lower acids and alcohols with a maximum chain length of 6 carbon atoms.

A study of changes in the composition of the skins of apples during maturation and storage failed to throw much light on the cause of scald. As fruit became more mature the wax, oil, ursolic acid, and cutin of the skin increased, making the skin more resistant to gaseous diffusion; yet it became less susceptible to scald. The higher oil content of the skin of late-picked fruit might be of benefit in removal of volatiles from subepidermal cells. Fatty esters of the oil fractions of the skin were produced most rapidly at the beginning of storage; subsequently these nonvolatile esters declined while the rate of volatile ester production increased. Thus the production of volatile esters could be linked with the accumulation of fatty acids in the oil fraction of the skin. Whatever the toxic substances are, they result in death of cells, lowered respiration of affected skin, and enzymatic browning accompanied by decrease in phenolic content.

OTHER DISORDERS CAUSED BY VOLATILE EMANATIONS

A lenticel spotting of certain varieties of apples was attributed to ethylene by Kidd & West (51). They were able to produce it by exposing apples held at 40°F. to ethylene in concentrations of 1 part to 500 or to volatiles given off by ripe apples. A spotting of Rome Beauty apples was controlled by use of oiled paper wraps and by air purification with activated carbon by Baker & Maxie (2), furnishing evidence that it was caused by apple volatiles capable of being removed by oiled wraps or activated carbon and therefore the volatile was not ethylene. Anjou pears develop a superficial type of scald more like apple scald than the usual form of pear scald which extends deeply

into the flesh and has a foul odor. Hartman (32) reported that this disorder could be controlled with oiled paper wraps, a fact which indicates that it is caused by volatiles capable of being absorbed by oiled paper.

VOLATILE EMANATIONS AND RESPIRATION

The extensive recent reviews of Smock (86), Biale (3), and Porritt (75) gave the background of the discovery, identification, and physiological activity of a number of compounds in the volatile emanations from several plant tissues. Since these reviews appeared, several detailed studies on the volatile emanations from apples were reported. These volatile compounds and their possible role in certain functional disorders of fruit during storage were discussed in the preceding section of this review. A number of volatile compounds were identified by using the newer techniques of paper chromatography. Mass and infrared spectra of apple volatiles were obtained by Turk, Smock & Taylor (94), Henze, Baker & Quackenbush (35), and Thompson (92), but the patterns were too complex to permit more than the identification of a few compounds such as ethanol and acetaldehyde and to indicate the presence of mixtures of esters, aldehydes and perhaps alcohols.

There is little evidence to indicate that the nonethylenic volatile emanations from fruits and vegetables influence respiration rates or accelerate ripening as does ethylene. The evidence is very conclusive that ethylene is a prominent constituent among the emanations from many plants and is related to important postharvest physiological responses of many fruits and vegetables. The advantages of the commercial usage of ethylene to hasten or alter certain physiological responses have been inconclusive in a number of instances although recent findings [Heinze & Craft (34)] have tended to clarify the conditions under which ethylene can be used advantageously.

Hall (28) recently studied the influence of different substrates on the production of volatiles as measured by direct absorption of the volatiles in potassium permanganate solution. Using crude extracts of apple juice and of *Penicillium digitatum* as enzyme preparations, Hall found that substrates of ethanol, arabinose, pectic acid, or pectin gave the highest yields of emanations which he termed ethylene. From this he postulated that ethylene may originate from simple sugars or organic acids and that the insoluble intracellular and cell-wall constituents such as pectin compounds and hemicellulose are degraded at a later stage by the respiratory mechanism of the climacteric. The hypothesis was based largely on the fact that pectin and its hydrolytic products form a good source of the emanations measured. Further evidence is needed to show that the techniques and crude preparations were indicative of ethylene production.

Biale (3) indicated that in most instances ethylene does not modify the normal respiratory behavior pattern except to shift the time axis. Biale found that nearly all the changes brought about by ethylene treatment were changes that would occur during the regular course of ripening.

Recently Biale, Young & Olmstead (4) studied the relation of ethylene

production to the climacteric rise in respiration in 14 species of fruits. With only one exception (mango) the evolution of ethylene was found to be associated with the climacteric, but no definite evidence was obtained whether ethylene production precedes or follows the onset of the respiratory rise. They advanced the hypothesis that ethylene is a product of respiratory change rather than a causal agent. The pronounced climacteric of the mango without any measurable production of ethylene is given as some evidence for the hypothesis. Additional evidence is cited in the response of avocado and cherimoya to chilling at 41°F. (5°C.) for 34 to 45 days. Chilling interfered with the ethylene production but had little effect on the respiratory process as measured by the rise in respiration immediately following the chilling period. In the chilling response Biale and co-workers do not differentiate between increase in respiration resulting from a change of temperature effect and a true climacteric associated with ripening.

Hansen (30) showed that ethylene production was not directly related to respiration in ripening pears. Respiration continued to rise with increase in temperature beyond 68°F. (20°C.). On the contrary, ethylene production declined rapidly, and at 104° (40°C.) no emanations could be quantitatively detected. Results obtained in U. S. Department of Agriculture laboratories at Fresno, California, show that some varieties of plums which normally fail to ripen at 90° also fail to produce ethylene at that temperature. Considerable varietal variation was found in this response, however all varieties ripened normally at 90° when treated with ethylene previously at a lower temperature.

Biale, Young & Olmstead (4) suggested that changes in metabolic reactions, more universal in nature than ethylene evolution, are associated with the chemical transformations characteristic of the climacteric. These suggestions are along the lines proposed earlier by Robertson & Turner (79) that the respiration rate in plant tissue may often be limited by the low concentration of phosphate acceptors available when the phosphorylations from respiration are more rapid than the dephosphorylations in syntheses. If the respiration in the preclimacteric is controlled by the concentration of phosphate acceptors then either an increase in phosphate acceptors or an uncoupling of the oxidation systems through the use of 2,4-dinitrophenol (DNP) should increase the rate of respiration. Pearson & Robertson (72) used small discs of apple tissue and observed the postulated results. DNP doubled the oxygen uptake on a sample taken seven weeks before the beginning of the climacteric and the addition of adenosine triphosphate increased the rates by about 30 per cent. After the climacteric, the addition of either compound caused only a slight or uncertain increase in oxygen uptake. A six-fold increase in succinoxidase activity in the extracts of climacteric apple tissue was also found to accompany the increase in respiration. More recently Millerd, Bonner & Biale (66) showed that particles of a mitochondrial nature isolated from preclimacteric and climacteric avocados respond to DNP in a manner similar to that reported by Pearson & Robertson (72) for apple

tissue. Addition of a phosphate acceptor, adenylate, increased the respiration of the particles from the preclimacteric but not from climacteric fruit. They suggested that a natural uncoupling agent may develop as the fruit ripens and this decreases the dependence of respiration on the phosphorylative system. Some evidence for the presence of a phosphorylation uncoupling agent was obtained from an extract of climacteric avocados. Addition of the extract to mitochondrial particles from mung beans caused an appreciable stimulation in respiration.

CHILLING INJURY

Many fruits and vegetables are injured physiologically by low storage temperatures considerably above the freezing point of the tissues. The most common symptoms are surface and internal discolorations, pitting, susceptibility to decay, and failure to ripen properly. The type and severity of the injury vary with the commodity and the environmental conditions. In some instances the injuries, such as wooliness in peaches [Boyes (5)], have symptoms that differ appreciably from those more commonly observed.

Factors other than actual temperatures during storage operate to enhance or modify the chilling effect. Species and even varieties of fruits and vegetables differ widely in their susceptiblity to chilling. Most of the varieties of apples grown in the United States can be held without injury at 31° to 32°F. whereas some, like McIntosh and Rhode Island Greening grown in the Northeastern states and Yellow Newtown grown in cool coastal climates of California, are injured by temperatures lower than 36° to 40°. Bananas are frequently injured after a few hours slightly below 55°. Fruit and vegetables of tropical or subtropical origin are considered more susceptible to low-temperature injury than temperate-zone plants but this is a broad generalization and is not without exception. Morris & Platenius (70) found that relative humidity strongly affected the severity of chilling injury as evidenced by pitting in cucumbers and peppers. Lutz (54) noted that curing of sweet potatoes had a great influence on their susceptibility to chilling. Wardlaw (97) found immature tomato fruit more subject to low-temperature breakdown than mature fruit. Kidd & West (47) noted that summer-grown tomatoes were much more resistant to injury and developed less wastage after low-temperature storage than autumn-grown fruit. Kidd & West (48, 50) found that apples in their respiratory climacteric are more susceptible to chilling injury than fruit in either a pre- or a postclimacteric phase.

Intermediate temperatures have, in some instances, given greater chilling injury than either higher or lower temperatures (1, 11, 16, 17, 65). Plums stored for 25 days at 37°F. showed greater injury than those at either 31° or 40° [Davies, Boyes & Beyers (17)]. Pitting of the rind of Marsh grapefruit after 3 to 4 weeks' storage is rarely found at 30° to 32° or at 50° to 55°, but intermediate temperatures frequently cause severe pitting [Brooks & McColloch (11); Davies & Boyes (16); Wiant et al. (99)]. The incidence of wooli-

ness in peaches is much greater at 37° than at either 31° or 45° [Davies, Boyes & de Villiers (15)]. The greater injuries noted at the intermediate temperatures are possibly restricted to a rather specific time period [Smith (84); van der Plank & Davies (95)] since the differences may be due to the more rapid appearance of limited injuries. After longer storage periods the injuries become more nearly inversely proportional to the temperature because the low temperatures result in the slow development of injuries of a more extensive and serious nature.

Interest has been centered for some time on the reactions that are involved in the chilling mechanism. Van der Plank & Davies (95) considered fruit at the time of storage to have an inherent primary susceptibility which predetermines a transition temperature above which fruit will remain healthy and below which it will be injured. The transition temperature does not necessarily remain fixed but may drift as the fruit weakens in storage. This shift of the transition temperature during storage is termed secondary susceptibility. The factors that predispose a fruit to secondary susceptibility are not necessarily the same as those which cause primary susceptibility. The amount of injury at any given time is dependent on an equilibrium factor which is based on the distance of the temperature below the transition point and a kinetic factor which regulates the rate of chemical change. The kinetic factor operates in the opposite direction from the equilibrium factor with change in temperature. By use of these two opposing factors van der Plank & Davies (95) explained the greater injury at higher temperatures as simply a more rapid manifestation due to the kinetic factor although greater injury was eventually noted at the lower temperatures.

Plank (73) has offered a somewhat more simplified explanation of the chilling mechanism. He assumed that two main types of reactions are involved in the cells, one leading to the accumulation of cell poison and the other to its removal. By selecting values for the temperature coefficients in his equations he was able to show the critical temperature at which the production and removal of poison are equated and below which cell poison would accumulate and manifest chilling injury.

Smith (84) raised an objection to any all-embracing theory because of the observed fact that there are at least several types of injury which can be symptomatically distinguished. Any temperature-injury curve must of necessity be very complex. Smith provided further evidence for the theory of toxic materials in his work with Victoria plums. Severe injury was obtained after storage at 31°F. for five weeks. When the storage period was interrupted after 15 to 20 days with a two-day period at 65° and the plums were further exposed to 31° for 15 or 20 days there was little or no injury. Kidd & West (49) found a beneficial effect of subjecting apples to brief warming periods which gave almost complete control of scald in susceptible varieties. In this case, it was suggested that the toxic substance was a volatile accumulated at cold storage temperature and expelled by exposure to warm temperature.

A dual temperature treatment of 5 to 10 days at 31°F. and the remainder of the storage at 45° or 50° gave the best control of wooliness in peaches [Kidd & West (49); Smith (85)] and has become a standard practice in the refrigerated transport of South African plums and peaches to the United Kingdom [Fidler (22)]. The effectiveness of the interrupted and the dual temperature treatments can be hypothetically pictured as due to the accumulation of a toxic or inhibiting substance which can be removed at a higher temperature if its accumulation has not proceeded too far. The idea of the accumulation of toxic substances has been prevalent for some time. Molisch (69) in 1896 described a severe brown spotting of some tropical plants after a short exposure to temperatures of 33.8° to 39.2°F. (1° to 4°C.) as a type of breakdown resulting from the accumulation of toxic substances in the cells due to incomplete oxidation. Nelson (71) proposed that the toxic material may be a fragment of a hydrolyzed glucoside and that unfavorable external conditions prevent the normal detoxification process.

Although the accumulation of sugars and organic acids and other slight changes have been noted in certain plant materials after a period of exposure to low temperatures, chemical analyses for the major constituents have generally failed to give any specific information on the mechanisms of chilling injury [Jones (42); Lorenz (52); Miller (62); Smith (85)]. Jones (42) noted a slight failure of chilled papaya to hydrolyze sucrose to reducing sugar but found a much more marked effect on the respiration as evidenced by the temperature coefficient at the lower temperatures. Chilling of sweet potato roots caused by freezing of the vines before harvest was found by Ezell, Wilcox & Crowder (19) to result in the failure of the roots to synthesize carotenoid pigments in any appreciable quantities during the subsequent curing and storage periods. The loss of the ability to synthesize carotenoid pigments after harvest was considered by these authors to be a sensitive measure of physiological injury in sweet potatoes. Chilling conditions before harvest had little effect on the changes in ascorbic acid content during the storage period. Miller & Heilman (63) suggested that the destruction of ascorbic acid constitutes the first phase in the development of low-temperature injury in pineapple. They proposed that interference with a specific step in the respiratory process causes quinones to accumulate because of their failure to be converted back to phenols by ascorbic acid and that the accumulation of the quinones results in the discoloration noted in many kinds of chilled fruits. Investigations in the authors' laboratories have failed to show any association of the concentration of ascorbic acid with chilling in tomatoes.

Low temperatures have a marked effect on the rate of respiration and it has been suggested [Jones (42); Nelson (71)] that chilling injury is associated with an abnormal course of respiration. Higher Q_{10} values were observed at the lower storage temperatures [Haller *et al.* (29); Platenius (74)] but Platenius (74) found no significant deviation in the respiratory quotient in

relation to chilling injury during the storage period when 10 different kinds of vegetables were subjected to a chilling temperature of 32.9°F. (0.5°C.). Many of the vegetables developed typical chilling symptoms during the tests. Biale, Young & Olmstead (4) noted that several kinds of fruit exhibit a fairly normal respiratory climacteric after having been subjected to chilling conditions but that the capacity for the production of certain volatile emanations was markedly restricted or eliminated. The failure of some fruits to ripen after being subjected to chilling temperatures could well be associated with a disruption of a mechanism for the production of volatiles.

Although there is some evidence that the respiratory system is affected there is no definite information indicating an inactivation or partial inhibition of any specific enzyme system or any accumulation of toxic metabolic intermediates associated with the chilling injury. The changes in carbohydrates during storage at low temperatures in some commodities show that the equilibrium in some enzyme systems is disturbed appreciably. The accumulation of organic acids also indicates a change in the course of certain metabolic functions but neither the carbohydrate nor the organic acid changes have been shown to have any direct relation to the chilling injuries. There is also the possibility that the initial stage in the chilling process may be more of a physical phenomenon involving permeability of intracellular membranes, but the lack of direct evidence does not justify an elaboration of this possibility.

GROWTH SUBSTANCES

Many reviews and extensive symposiums have been concerned with various aspects of the effects of growth substances on plants or plant tissues. In this section brief attention will be given to those growth substances and other chemical compounds that have been shown to have a pronounced effect on the storage life of fresh fruits and vegetables, on the diseases that develop in these stored products, and on the ripening of detached fruits.

The application of certain growth substances such as 2,4-dichlorophenoxyacetic acid (2,4-D) to harvested lemons and limes before storage has been shown to affect markedly the retention of the buttons in a green condition, thus preventing the undesirable blackening of buttons that frequently develops during storage [Gates (25); Kessler & Allison (45); Stewart (91)]. Comparable results were obtained when the fruit on the tree was sprayed with these chemicals before harvest. Some oranges and grapefruit were also found to have better keeping qualities after treatment with 2,4-D [Stewart (91)]. Miller & Marsteller (64) found that pineapples sprayed with parachlorophenoxyacetic acid 10 days before harvest retained more ascorbic acid and showed less physiological breakdown after exposure to relatively low temperatures than unsprayed fruit.

Many publications dealing with growth substances in relation to the

control of fruit drop have appeared in recent years. It is generally agreed that the application of growth substances to apples, pears, and peaches for the control of fruit drop has in many cases the added effect of hastening maturity. This hastening of maturity plus the tendency to leave the fruit on the tree longer may have a considerable effect on its subsequent storage life [Gardner (24)]. It has been shown that some of the stimulation of ripening and respiration caused by the growth substances can be counteracted in appies by the simultaneous application of maleic hydrazide [Smock, Edgerton & Hoffman (87)].

The keeping quality of several fresh vegetables is affected by the application of growth substances. Prevention of abscission of leaves in stored cauliflower by treating the freshly cut heads with the methyl ester of α-naphthaleneacetic acid or with either of several forms of 2,4-D was demonstrated [Carolus, Lee & Vandemark (12); Hruschka & Kaufman (36)]. Preharvest applications were also found effective in preventing abscission of the leaves. Marth (59) found that the loss of green color and the shedding of florets in broccoli was very markedly retarded by the application of 2,4-D or 2,4,5-trichlorophenoxyacetic acid (2,4,5-T) to the freshly harvested heads. The 2,4,5-T compound was the most effective. The application of 4-chlorophenoxyacetic acid to snapbeans a few days prior to harvest caused a better retention of the original color and greatly inhibited the loss of moisture from the pods [Mitchell & Marth (68)].

The control of sprouting of several vegetables during storage has received considerable attention. Among some of the compounds found most effective for treating harvested potato tubers prior to storage are the esters of naphthaleneacetic and trichlorophenoxyacetic acid. More recently the carbamates, particularly 3-chloro-isopropyl-N-phenyl carbamate, were shown to be very effective when compared with other sprout-inhibiting chemicals on potatoes [Marth & Schultz (60); Rhodes *et al.* (78)]. Other compounds used to some extent are 2,3,5,6-tetrachloronitrobenzene [Luckwill (53)], and maleic hydrazide [Kennedy & Smith (43, 44)]. Maleic hydrazide and some of the phenoxy compounds have been used as sprays on the potato plants at various periods during the growing season with a considerable degree of control of the sprouting of the tubers during the subsequent storage period [Ellison & Smith (18); Kennedy & Smith (44)]. The sprouting of onions has also been inhibited by the application of maleic hydrazide to the green plant prior to the harvest of the bulbs [Johannessen & Oebker (41); Wittwer & Sharma (100)]. In contrast to many of the growth substances maleic hydrazide appears to have an inhibiting effect on the respiration of the plant [Smock & Southwick (88)]. This inhibition may act through some of the dehydrogenase systems [Isenberg *et al.* (40)].

In a few instances growth substances have had an effect on diseases in stored fruit. The incidence of *Alternaria* rot, usually starting at the buttons in lemons and limes, was reduced by growth substances that held the buttons in a greener more vigorous state [Kessler & Allison (45); Stewart (91)].

Scald in pears and apples was reduced to some extent by treatment with several of the growth substances or by combinations of some of them, but an explanation for this action is lacking [Hansen (31); Schomer & Marth (82)].

The ripening of detached fruit by treatment with growth substances has received some attention. Mitchell & Marth (67) found that 2,4-D applied as a spray or dip to harvested fruit stimulated the ripening of bananas, apples, and pears but had no discernible effect on fruit with low starch reserves such as tomatoes, peppers, and persimmons. It appears that the diastatic activity in the high-starch fruit is accelerated along with a conversion of insoluble pectin to the soluble forms, which is indicated by the decrease in resistance of the tissue to pressure. As might be expected, the increased rate of ripening caused by 2,4-D in bananas, apples, and pears is accompanied by a subsequent increased rate of spoilage [Culler, Weiser & Witman (14)]. Harvested oranges were unaffected by 2,4-D application [Culler, Weiser & Witman (14)].

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PHYSIOLOGICAL ASPECTS OF FUNGUS DISEASES OF PLANTS

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The present review will be concerned with the inception and results of diseases of higher plants caused by obligately parasitic fungi. The term "obligate parasite" refers to those fungi which have not yet been cultured even in their vegetative phase except on a living host plant and whose nutrient requirements and physiology are therefore largely unknown. Particular emphasis will be placed on diseases caused by rusts (Uredinales) and powdery mildews (Erysiphaceae), but occasional reference will also be made to other obligate parasites, particularly the downy mildews (Peronosporaceae).

Some physiological aspects of these diseases are included in recent reviews (1, 2), and the literature on variation in rusts has been reviewed by Johnson (3). Gäumann's *Pflanzliche Infektionslehre* (4) is an extensive and provocative collation of many aspects of plant diseases, and is available in English translation (5), and in a second edition in German (6). In a monograph on *The Cereal Rusts*, Chester has presented a comprehensive survey and analysis of the litereature on stem rust of wheat and some allied diseases (7), and Volume VII of Buller's *Researches on Fungi*, published posthumously, has many interesting observations on certain aspects of the biology of the Uredinales (8). A monograph in Russian is concerned primarily with the author's investigations on the metabolism of plants infected with obligate parasites (9).

The terminology used to describe attributes of host and parasite has been reduced to a minimum for purposes of this review. "Susceptibility" and "resistance" will be used to designate conditions of the host which, respectively, favor or do not favor development of a parasite. Plants showing extremes of these conditions will sometimes be referred to as "congenial" and "immune," respectively. "Virulence" and "avirulence" will designate the complementary conditions of the parasite. The term "infection" will be used to designate the penetration of a host cell and whatever subsequent progress the parasite makes.

The literature on these diseases will be considered in three sections corresponding to three aspects of the origin and development of disease: (*a*) the inception of disease, (*b*) environmental factors affecting the host's suitability as a substrate for development of the parasite, and (*c*) changes in host physiology resulting from infection.

The Inception of Disease

Genetic basis of host restriction.—A given plant species is liable to attack only by certain parasites, but these parasites may come from the most diverse groups of the plant and animal kingdoms. A particular species of par-

asite however, tends to be limited to a more or less narrow spectrum of host species. Thus, a bean plant may be attacked by rusts, mildews, anthracnose, and many other fungi, and by viruses, insects, and diverse bacteria, but *Uromyces phaseoli* occurs only on bean. This kind of restriction in host choice is particularly marked amongst obligate parasites. It is, in fact, impressively narrow when one examines the hosts which may be parasitized by members of a clone instead of a species, for the clone may be capable of infecting only certain members of a variety of host plant and not the others. This fact has been epitomized in the physiological races of rusts and mildews, and is perhaps responsible for an overemphasis on the side of host uniqueness. Some recent papers have pointed out that even the highly specialized physiological races which will not attack some members of the preferred host species, may nevertheless attack members of other species or even of other quite widely separated genera (10 to 13). There is thus a highly specific determination of resistance and susceptibility operating within the limits of a less precise restriction to a group of host species. The specific determination has a demonstrable genetic basis, resistance or susceptibility to a particular clone of the parasite depending often upon a single gene. Virulence and avirulence in the parasite also have a genetic basis (3), so that the susceptibility of a potential host plant must be defined in terms of the genotype of the parasite. The significance of the host-gene: parasite-gene relationship for susceptibility is emphasized by the findings of Flor, whose careful and extensive studies with flax rust (*Melampsora lini*) have shown that for each pair of alleles controlling resistance and susceptibility in the host there is a corresponding gene pair controlling virulence and avirulence in the parasite (14 to 17). If flax possessed a single gene controlling resistance and susceptibility, the inheritance of virulence in the parasite behaved as though it were controlled by a single gene. The host was infected by a rust only when the flax had the requisite alleles for susceptibility and the parasite had the complementary alleles for virulence; the host remained resistant to a parasite with the gene for avirulence. If the flax possessed more than one gene whose alleles controlled resistance and susceptibility, the inheritance of virulence in the parasite was also controlled by a corresponding number of genes for virulence. The genetically susceptible host was actually infected only by a rust whose opposing genes all made for virulence. Susceptibility requires, therefore, a more exacting correspondence between host and parasite genes than does resistance, since the plant is resistant either by virtue of its own genes for resistance or, alternatively, through lack of any one of the genes for virulence in the parasite. These findings correspond with the general observation that susceptibility to a given parasite is the exceptional, resistance the more widespread property (4, 18, 19). They imply that susceptibility results from an interaction between specific gene products of the host and of the parasite and are in accord with the view that susceptibility is a positive attribute of the host and not dependent solely on the initial absence of materials toxic to the fungus (20).

Flor also pointed out that a relatively small number of genes controlling resistance in a few differential hosts would create the possibility for a very large number of physiological races, and he actually found in the F_3 generation from a single cross of *Melampsora* 64 distinct races of which 62 were new (16). Although the physiological race has been of historical and practical interest, it is an arbitrary unit and should not be regarded as fundamental in analysis of host-parasite relations (7).

Development of spores on artificial substrata.—The inception of fungus diseases usually starts with a spore. Reserve materials in the spore are sufficient in most cases to support the initial development of the parasite before the host nutrients become available. The infective spores of rusts and mildews will germinate on a variety of artificial media, or on water or solid surfaces containing no nutrients. On a synthetic nutrient agar Hurd-Karrer & Rodenhiser (21) obtained development of appressoria and substomatal vesicles similar to corresponding structures formed on host plants from uredospores of six species of cereal rusts. These occurred on about 0.1 per cent of the spores germinating. Infection hyphae developed from the vesicles in some cases. Structures which fused with other germ tubes, and in a few cases gave rise to further hyphal outgrowths, were also observed on synthetic media containing glucose and mineral salts, but did not appear in the absence of nutrients (22). Sharp & Smith (23) reported that formation of appressoria, vesicles, and infection hyphae could be obtained with high frequency (up to 40 per cent) from uredospores of *Puccinia coronata* if several p.p.m. zinc was added to gelatin media at pH 6.2 to 6.6. Dickinson, investigating germination of rusts and powdery and downy mildews on artificial membranes, has shown that with suitable membranes the germ tubes will grow along the surface and form structures characteristic of each type of spore as it develops on a leaf (24, 25, 26). Penetration of membranes occurred in some instances and structures resembling haustoria formed beneath the membrane (26). Development up to and including the formation of haustoria is therefore dependent only upon the presence of simple nutrient substances such as sugar and inorganic materials, and the guidance of the growing germling is perhaps further aided by the physical and chemical nature of the surfaces which it encounters.

Development on uncongenial plants.—In search of an explanation for the failure of these parasites to develop except on certain hosts, careful investigations were made, beginning in the early part of the century, of the stage of development reached by spores sown on unnatural (not related to the congenial host species) and resistant hosts. The studies of Ward (27) and Gibson (28) with rusts, and of Salmon (29) with powdery mildews all showed the same general picture, which has been confirmed and extended by subsequent investigations of these and other obligate parasites. Germination of the spores and development of characteristic structures of the parasite occur on the surface of many plants far removed taxonomically from the natural host, as well as on closer relatives and resistant members of the preferred host

species (18, 19, 30 to 34). So also does the correlated growth of the germ tube which is required to bring the hyphae to the site of penetration (18, 30, 33, 35, 36, 37). Such conditions as are needed for this much development are provided by most plants, and an active exclusion usually comes into play only after the barrier of the cell wall has been penetrated (10, 18, 19, 31, 32, 37, 38). Particularly on resistant plants closely related to congenial hosts, considerable development of mycelium may be permitted before the aspiring parasite is finally stopped.

Where penetration occurs, and occasionally even without penetration of a host cell (31), the parasite elicits marked responses from the invaded cell. Changes in the staining reactions of both host and parasite occur at once, whether the host is congenial or not (25, 31). The haustoria of many obligate parasites become associated with or actually enveloped by the host nucleus (39, 40, 41), but this has rarely been seen with powdery mildews. Changes in appearance and composition, followed by death of the attacked cell, occur in many immune and resistant plants, either before or after penetration (18, 30, 31, 37, 42). In others, death of the host cells is delayed and some myce-lium develops, but more host cells are eventually involved in necrosis. The resulting hypersensitive flecks are characteristic of immunity or resistance to many obligate parasites. They do not invariably occur on resistant plants, however, as arrest of the parasite can occur without sacrifice of host cells. In a susceptible plant the development of an obligate parasite runs its full course without killing the host cells. When death eventually results from such infections, it is not a few cells which die but an entire organ or the whole plant.

Approaches to the chemical basis of resistance.—Information concerning the nature of host substances operating in the initial protection against in-vasion has been sought in three ways: (*a*) by grafting experiments, which have been reported in only a few instances and have failed to show any effect of a resistant stock upon a susceptible scion (4, 28), (*b*) by attempts to cor-relate the toxicity of host extracts with resistance, and (*c*) by a search for specific types of compounds whose presence might be correlated with resist-ance.

Experiments with host juices have usually involved tests of their effects on spore germination, and have failed generally to establish any correlation with resistance even when precautions were taken to avoid the pitfalls in-herent in this approach (10, 43, 44, 45). These include loss of active sub-stances, production of spurious toxins during preparation and testing of an extract, modification or neutralization of activity by other substances in the extract, differences in activity against germination as compared with growth, and, in the case of rusts at least, interaction between substances in the ex-tract and self-inhibitor from the rust uredospores (46).

The third approach has produced some evidence that phenolic substances play a part in protecting plants against infection by rusts (43). Recently, a direct correlation between catalase activity and susceptibility to certain rusts

has been reported (47), indicating a possible role of hydrogen peroxide in resistance. Substances of this sort, which are of widespread occurrence in plants, may contribute to the broad basis of resistance, but cannot account for the specific differences between resistant and susceptible genotypes of the same species (7). Recent experiments using paper chromatography have revealed striking differences in composition between organisms with a single gene difference (48). This technique might help to indicate the classes of substances most likely to repay further study.

Effect of Host Environment On Development of the Parasite

Although the genotype of a plant determines its potentiality for becoming host to a virulent parasite, the realization of this potentiality is greatly influenced by environment. The conditions prevailing before and after infection both affect the physiology of the host and hence its capacity for excluding or for supporting development of the parasite. By appropriate treatment, a susceptible host may be made completely immune, a host of intermediate susceptibility may be shifted in either direction, and resistant or immune plants may become less resistant. High resistance is least readily modified, an intermediate reaction most readily (49 to 52). A particular treatment may be expected to have comparable effects upon different hosts only when the factor limiting development of the parasite is the same in both. In a resistant and a susceptible plant, the limiting factors are unquestionably different.

Effect of mineral elements.—High nutrient levels are generally considered to favor the development of obligate parasites by increasing growth of the host. Favorable effects upon several powdery mildews were obtained either by increasing the total salt concentration in Hoagland's nutrient solutions, or by decreasing the moisture content and hence increasing the total solute concentration in soil cultures (53). Of the elements taken in through the roots of the host plant, nitrogen has usually been found to enhance and potassium to reduce the development of obligate parasites. There is less agreement concerning the effects of phosphorus, but it has more often been reported to have adverse effects on the parasite. However, in recent field experiments, Smith & Blair found that phosphate enhanced mildew infection while nitrogen had no effect (19). They attributed these results to an increase in growth rate of the host following phosphate but not nitrate fertilization. There are other recent reports that mildew (10, 54, 55) development is not favored by higher levels of nitrogen even when supplied as nitrate. When supplied as ammonium ions, nitrogen may be detrimental or not, depending upon other conditions. Gassner & Hassebrauk (56) found that ammonium nitrogen was detrimental to leaf rust in weak light (as it is for the host plant also) but favorable in stronger light or when carbohydrate was fed together with the ammonium salt. Unfavorable effects of $(NH_4)_2 SO_4$ compared with KNO_3 were also found by Daly (55).

Further evidence that the growth response of the host plant is of more importance than the nature of the element supplied comes from the experi-

ments of Last with powdery mildew (57). Using as criterion the number of colonies developing per cm.^{3} of leaf surface per day, he has shown that the response to nitrogen fertilization is directly correlated with the rapidity of growth which ensues, and persists only as long as the increased growth rate. A close correlation between mildew development and rate of formation of new leaf surface was obtained when growth was induced by the new addition of nitrogen to previously deficient plants at 14°C., or when it was induced by transferring plants already supplied with different levels of nitrogen from 7° to 14°C. Unfortunately, Last's criterion for mildew development summarizes three separate processes (spread of spores, success of infection, and development of colony) in one figure. Nevertheless, these results provide additional evidence that active growth, however it is induced, is a major factor predisposing the host to infection. It is to be hoped that there will be experiments showing which phase of mildew spread is affected by growth rate of the host.

Attempts have been made to explain the effects of an excess or deficit of particular elements in terms of the resulting changes in host composition, and thus to discover some class of tissue component which could be correlated with susceptibility. As Brooks (58) has pointed out, this is a difficult task to accomplish on the basis of experiments on mineral nutrition, and the conclusions drawn by different investigators have been conflicting. Although there may be many reasons for the frustration of this approach, two are particularly worth mentioning. One is the impossibility of relating presence or absence of a single component of a host to resistance or susceptibility in general, since one and the same host will be highly resistant to one race and simultaneously susceptible to another race of the same parasite. If a relationship exists, for example, between some nutrient in the host and susceptibility, it will only be evident when comparisons involve a genetically susceptible host infected with a genetically virulent parasite. A second point which must be kept in mind in trying to correlate host composition and susceptibility is the fact that accumulation of a substance which results from more rapid formation may have a quite different effect upon the parasite than a similar accumulation resulting from failure of the host to metabolize the substance further. Thus, for example, low nutrient levels of nitrogen compared with potassium lead to the accumulation of soluble carbohydrates, which are not used as fast as they are formed. Under these conditions the development of rusts (56) and mildews (59) is poor. On the other hand, carbohydrate feeding, which also leads to an increase in the content of soluble carbohydrates, favors the development of rusts (56, 60) and powdery mildews (61) on plants or leaves previously well supplied with mineral nutrients.

Pronounced effects upon the development of obligate parasites are induced by addition of lithium or cadmium salts to the soil or nutrient solution several days before inoculation. Wortley's report that the failure of mildew to develop on plants grown in soil with lithium was due to failure to penetrate the cellulose wall (62) has been confirmed by Smith & Blair (19). Kent (63) found an inverse correlation between lithium content of the leaves and mildew in-

tensity, but he left open the question of whether the effects of lithium were direct or through a modification of host physiology. Lithium concentrations in the leaf which reduced mildew growth had no effect upon rust. Cadmium added to soil or nutrient cultures will also confer resistance to mildew, and its effects were considered by Sempio (64, 65) to be connected with the appearance of refractile granules in the epidermal cells and with a change in the metabolism of cadmium-treated plants. Using the same varieties of wheat that Sempio used, as well as some additional ones, Meyer (66) found similar effects of cadmium, but could not correlate resistance to mildew with the occurrence of granules in the epidermal cells. The effects of cadmium were slight in the summer but pronounced at other seasons. A large percentage of the spores sown on plants with cadmium-induced resistance did not develop beyond the stage of haustorium formation, but the small percentage which were not arrested at this stage developed to the same extent as colonies on untreated hosts. The cadmium content of resistant leaves was found to be high enough to inhibit germination of mildew spores, but since the same amount of cadmium in the leaf reduced mildew development in some varieties and not others, she thought the effects of cadmium must be indirect. This might, however, depend upon differences in distribution of cadmium within the leaf in different varieties. In the same paper Meyer reports that zinc appeared to enhance mildew infection, whereas mercury had no effect, even at concentrations which damaged the host. Applications of borax to soil have been reported to prevent the developemnt of flax rust by inhibiting the fungus in the early stages of development (67).

Effect of carbohydrate supply.— A plentiful supply of carbohydrate to the host is a *sine qua non* for the development of obligate parasites on a genetically congenial host plant or leaf. This supply may come from the seed reserves, from photosynthesis, or from an external supplement provided by floating detached leaves on solutions of carbohydrates (60, 61, 68, 69, 150). With detached leaves the development of the parasite is proportional, over a wide range of concentrations, to the amount of soluble carbohydrate supplied (up to concentrations equivalent to an osmotic pressure of about twenty atmospheres). This relation to carbohydrate supply is one of the most secure generalizations that can be made about the requirements for development of obligate parasites, and has therefore been subjected to particularly careful scrutiny to determine exactly how far one can go in replacing the photosynthetic production of sugar by administration of carbohydrate solutions. When detached susceptible leaves are inoculated with rust or mildew, penetration and some development of mycelium occurs in light or in darkness with or without a supply of carbohydrate. Continued development of the parasites will occur in light with or without sugar but in continuous darkness only if sugar is provided (60, 61, 69). Glucose, fructose, sucrose, and melezitose are particularly favorable, but most other carbohydrates including galactose and starch are to some extent effective, as are some alcohols which have been tested (61). When supplied with these compounds the detached

leaves absorb and use them, remaining green and healthy in appearance for one to two weeks. Sempio (150) made the interesting observation that sorbose, alone of several mono- and di-saccharides tested, permitted maintenance of the healthy green color of detached leaves, without at the same time supporting any development of rust (*Uromyces appendiculatus* on bean). The condition of the leaf at the time of detachment has some influence on the subsequent development of the parasite. Young leaves of clover, still enlarging at the time of excision, supported better growth of mildew when floated on sucrose solution than older leaves treated similarly (68).

Effect of light and photosynthetic capacity of host.—The dispensability of the photosynthetic apparatus of the host is indicated by mildew development on excised etiolated leaves floated in the dark on sucrose solutions (61), by the development of rust pustules on albino corn (71) and barley (72), and by the observation recorded by Grainger (54) of mildew (*Podosphaera oxycantha*) growing on white leaves of hawthorne. Cutter (73) arrived at somewhat different conclusions from his experiments with albino corn seedlings. He grew albino plants in sterile culture with 2 per cent glucose supplied to the root system, and found no more than hypersensitive flecks and these only in the light. He also inoculated variegated geranium leaves with basidiospores of *Puccinia polygoni amphibii* and obtained pycnial lesions only on the green and not on either the white or the virescent areas of the leaf. The development of rust on some albinos and not on others suggests that something besides carbohydrate may be lacking in these plants, but the conclusion that it is chlorophyll seems unlikely, since viable spores were produced on albino barley (72) but no signs of rust appeared on virescent geranium leaves (73). Wu's observation of better rust development when sucrose was supplemented with yeast extract also points to a deficiency of something other than chlorophyll (72), but the supply of carbohydrate may have been the limiting factor in Cutter's experiments.

Experimental work on the relations between carbohydrate supply to the host and the development of obligate parasites originated with studies showing that rust developed on plants kept in the light but was suppressed as long as the host was kept in continuous darkness. The work of subsequent investigators showed that a sojourn in the dark, provided it was not too prolonged, delayed but did not prevent eventual appearance of sporulating colonies of the parasite (60, 61, 74). Deprivation of light during the initial few days after inoculation causes less delay and interferes less with the development of the parasite (white and brown rusts, downy and powdery mildews) than deprivation at later stages (70, 74 to 77). According to Sempio, the intensity of disease and development of the parasite are actually increased (over controls with normal day and night) by incubation in darkness during the first few days after inoculation, followed by a return to normal day conditions. Hassebrauk found a similar effect of darkness, but only with moderately resistant and not with susceptible plants (51). Cherewick (10) reported no effect upon powdery mildew development of continuous illumination or of a reduction in

the daily period of illumination to seven hours when these treatments followed inoculation, but a reduced development occurred if either treatment was started four days before inoculation. Sempio, however, (64, 78) obtained poorer development on plants exposed to continuous light following inoculation. Forward (74) found that the light period could be reduced to as little as 4 hr. without noticeably interfering with the development of stem rust. In a careful study of the question of light effects, Pratt (79) has shown that the minimum daily period of illumination required for normal development of wheat powdery mildew depends upon temperature. At 10°C., 3 hr. is sufficient. At 20°C., 6 hr., and at 25°C., 12 hr. are required. He found no significant effect of continuous illumination, and showed that in continuous darkness germination, penetration, and mycelial development are the same as in light up to the second or third day, when haustoria are formed and a sparse mycelium has developed. At 10°C., development in darkness was interrupted at a slightly earlier stage than at 20° or 25°C. Some inhibition of spore germination by visible light occurred at all wave lengths tested except 436 mμ. One minute exposure to ultraviolet (365 mμ) stimulated germination of "dry" spores (about 60 per cent water) but inhibited markedly after the spores had begun to germinate, as did longer exposures before or after the start of germination. Sempio, however, reports (80) no appreciable effect of continuous white light on spore germination or hyphal growth. Blue light (including some ultraviolet) is definitely less favorable than longer wavelengths for development of downy mildew (*Peronospora*) of lettuce (70).

Because of the wide variety of treatments employed in different investigations, it is difficult to reconcile completely all of the reports on the effects of light. In many experiments direct effects of temperature or radiation upon spore germination and infection contribute to an eventual reduction in development of the parasite, and the conclusions reached are influenced by the criterion used in assessing the effects of light. Taking these facts into consideration, it appears that illumination during the very early stages of infection has little if any favorable effect upon establishment of the parasite, but thereafter a certain minimum daily illumination is essential. The duration of the light period required is increased when metabolic activity is high or when photosynthesis is reduced (at high temperature and in the later phases of the disease). In view of the influence of photoperiod upon vegetative growth of many higher plants, it would be interesting to know whether host susceptibility is influenced by induction to flowering. The question could be readily tested with plants which require only a few days' exposure to the flower-inducing photoperiod.

Effect of temperature.—By appropriate manipulations of the temperature, the extent of development of many obligate parasites may be so influenced as to convert a normally resistant host into a congenial host and vice versa, these changes occurring within a range of temperatures which are suitable for the germination of spores and germ tube growth or zoospore discharge and

germination. The literature on this subject, particularly as it concerns the rusts, was recently reviewed by Hart (2). Of particular interest are those investigations in which variable temperature treatments preceded inoculation and the development of the parasite than followed under uniform conditions. Pratt (81) found that powdery mildew conidia might germinate at 30°C. but did not penetrate the host. If exposed to 30° for a few days and then inoculated at 20°, infection was indistinguishable from that of plants kept continuously at 20°. Last (57) similarly found no difference in mildew development on detached leaves at 20° whether the plants from which the leaves came had been at 7° or at 20° during the three days preceding detachment and inoculation. However, if similarly pretreated leaves were inoculated and incubated at 7°, more colonies appeared and they developed more rapidly on the leaves which had been at the higher temperature before inoculation.

Tapke (52) has reported experiments with moderately susceptible barley and wheat plants showing that their susceptibility to powdery mildew is determined by the growth conditions prior to inoculation. Plants grown under different conditions of moisture, light, and temperature, and then kept under uniform conditions after artificial inoculation were most susceptible if the leaves were "succulent." The greater susceptibility of wilted plants, earlier ascribed to their condition at the time of inoculation, Tapke ascribed to the previous conditions of growth. The wilting is only incidental to other more important physiological differences, as Sempio also pointed out (80).

Influence of growth substances in host.—The influence of growth substances in the host upon development of obligate parasites was first investigated by Pryor (82). The development of powdery mildew was determined on detached leaves floated on solutions of sucrose. Cucumber leaves sprayed with 0.01 p.p.m. thiamine showed an increase in number of colonies and rate of mycelial growth when compared with excised leaves from untreated plants. When thiamine was administered to the leaves after excision, 1.0 p.p.m. gave the best response, but in no case were the differences very great. Gottlieb & Hart (83) found no effects of thiamine, riboflavin, nicotinic acid, ascorbic acid, or indole acetic acid ($10^{-4}M$ and $10^{-6}M$) on *Puccinia graminis tritici* or *Puccinia graminis avenae*. Compounds were administered by four different methods to several different hosts. In a series of investigations on the action of sulfonamides on rust development, Hassebrauk (84, 85, 86) has found that several of these compounds will inhibit rust development without injuring the host. The inhibition of *P. triticina*, *P. simplex* and *P. coronata* by *p*-aminobenzosulfonoxy-methylamid-N⁴-D-glucoside sulfonic acid (ladogal) can be reversed by comparable concentrations (0.1 to 1.0 per cent) of *p*-aminobenzoic acid (PABA) when administration of PABA follows within eight days of the administration of sulfonamide. Reversal of inhibition by some other sulfonamides is likewise effected by PABA. PABA alone has no effect except for a slightly accentuated necrosis on resistant varieties. Hotson has reported counteraction of the sulfadiazine (0.03 per cent spray with 1.0 per cent Tween 20) inhibition of *P. graminis tritici* by PABA and also by folic

acid (87). The sulfonamides clearly do not kill the fungus which is first allowed to become established in the host tissues, but arrest its development until PABA is administered. These results are consistent with the hypothesis that PABA is an essential growth factor which must be provided to the rust from the host tissues (86), although the concentrations of sulfa compound required to inhibit are rather high for a PABA heterotrophic organism. The use of competitive inhibitors for studying growth factor requirements of obligate parasites has interesting possibilities, but cannot provide conclusive evidence for a growth factor requirement.

An inhibition of stem rust development by spraying the host with 0.1 per cent of the butyl ester of 2,4-dichlorophenoxyacetic acid (2,4-D) has been reported (88) but the concentration required is high and the action is almost certainly a direct inhibition of spore germination. Uredospore germination is inhibited by 0.005 per cent of this ester (88) and *Puccinia coronata* uredospores by 0.2 to 0.3 per cent of the isopropyl ester (89). Occasional pustules which do develop on plants sprayed with 2,4-D are normal.

Metabolism and permeability of host.—The metabolic aspects of resistance and susceptibility are implicit in much of the work reviewed above, but they have been explicitly analyzed primarily by Sempio. He distinguishes between two sources of metabolic resistance, one depending upon changes in the relative rates of major metabolic processes, the other upon diversion of host metabolism into channels which do not lead to the intermediates required by the parasite (80, 90). It is not, however, clear that the two are actually distinct. His investigations with all of the major groups of obligate parasites have led to the hypothesis that relatively high rates of photosynthesis and of glycolysis and a low rate of respiration make for resistance, whereas relatively high respiration makes for susceptibility (see 80, 91 for recent summaries). He has measured the over-all rates of respiration (as O_2 consumption), glycolysis (as anaerobic CO_2 production), and photosynthesis (as CO_2 utilization in light) in healthy plants after various treatments which affected susceptibility to obligate parasites, and found that susceptibility increased if the treatment led to an increase, and was lowered if it led to a decrease in the relative rate of respiration (76, 80). The absolute rates of O_2 consumption were not appreciably altered by most treatments, but anaerobic CO_2 was reduced. Changes in the relative rates of these processes during the development of the disease, whether produced by the action of the parasite alone or by additional experimental treatments such as removal of light, are in harmony with the hypothesis. The ratio, aerobic CO_2 production/anaerobic CO_2 production, is similar to the ratio measured by Sempio, and is an index to the occurrence of a Pasteur reaction when anaerobic CO_2 arises from alcoholic fermentation. If the ratio before treatment is less than 3.0, aerobic inhibition of carbohydrate breakdown is indicated, and when treatment increases the ratio appreciably, a removal of the aerobic inhibition of glycolysis has occured. Sempio's data show mostly a decrease in anaerobic CO_2 production and little change, or an actual reduction in O_2 uptake by treatments which favor

development of the parasite (76, 80). The treatments do not, therefore, lead to any increase in the intermediates available at the time of inoculation. By facilitating such an increase after infection and the accompanying mobilization of carbohydrates, however, they may make an important contribution to the intermediates available to the parasite during its development (see next section). Carefully planned experiments, including measurements of aerobic CO_2 production, are needed to establish definitely whether or not susceptibility is generally associated with an inhibition of the Pasteur reaction. The marked enhancement of rust development produced by chloroform (20) might be explained in this way, since chloroform inhibits the Pasteur reaction and accelerates respiration.

Thatcher has proposed that the permeability of the host cells plays a determining role in making nutrients available for the development of obligate parasites (92, 93, 94). He pointed out that the increase in susceptibility produced by such treatments as hardening to cold and narcotization with chloroform was associated with increased permeability, as measured by the time required for deplasmolysis in hypertonic solutions of urea. The decrease in permeability caused by rusts in resistant hosts was nearly counterbalanced by chloroform treatment, and there was a correspondingly more susceptible reaction in such hosts (93). Changes in permeability were also considered by Humphrey & Dufrenoy to play an important part in providing the parasite with suitable nutrients (95).

Effect of infection with other parasites.—The infection of a plant by one parasite has often been observed to influence its susceptibility to a second. High susceptibility to leaf rust induced by powdery mildew on normally rust-resistant hosts has been reported in several instances (96, 97, 98). The converse, a greater development of mildew on old bean leaves which are normally resistant, has also been noted, as well as marked effects of infection in one leaf upon the course of disease in the opposite primary leaf of bean plants (99). According to earlier reports, one race of rust had no effect upon the development of another, but recent investigations show that inoculation with mixed races is sometimes followed by a more or less rapid increase in one race at the expense of others (100, 101). Straib (49) and Parker-Rhoades (44) reported that plants infected with bunt (*Tilletia* sp.) became much more susceptible to various rusts, whereas Thatcher (94) found no such effect of smuts (*Ustilago* sp.). On bean leaves infected with tobacco mosaic virus, rust development and sporulation are reduced (102, 103).

Certain kinds of local injury may be sustained without preventing development of the parasite, or may even enhance it. Thus, by careful treatment, Dickinson was able to obtain luxuriant aerial mycelium of *P. graminis* growing out of mesophyll tissue exposed by stripping off the epidermis (149). Development of *Erysiphe graminis* on the exposed mesophyll of resistant leaves was reported earlier by several investigators (29, 38, 104).

Development of the parasite on host tissue cultures and artificial media.—One of the greatest handicaps in many kinds of studies with obligate para-

sites is the difficulty in maintaining absolutely pure cultures. The solution of this problem has been sought by three approaches: two-membered cultures on living host leaves or seedlings, two-membered cultures on host tissue cultures, and saprophytic cultivation.

The method of cultivation of obligate parasites on excised leaves has been widely and profitably used (1). It may be refined by appropriate precautions to give cultures free of contamination, but such cultures must be continually renewed by sterilization of fresh leaves. Seedlings may also be grown aseptically and pure cultures of obligate parasites obtained and maintained in this way. Recent developments in techniques of cultivating higher plant tissues have opened up the possibility of cultivating the host and parasite in sterile cultures where the two members of the association can be transferred indefinitely. The method affords opportunities for studying the influence of cultural factors, particularly organic nutrients, not open through other techniques. Morel (105) first reported successful establishment of downy mildew on grape callus cultures and later described extensive investigations resulting in the cultivation of some other obligate parasites on plant tissue cultures (106). Attempts to establish rust cultures by spore inoculations were not successful. Such cultures were subsequently obtained by Hotson & Cutter (107) starting with surface sterilized tissue pieces of juniper infected with *Gymnosporangium juniperi-virginianae*. Growth of both organisms was slow, but cultures of the complex were carried through several subcultures. Further observations on such cultures have been noted by Hotson (108). Heim & Gries (109) obtained some growth of *Erysiphe cichoracearum* on sunflower tumor tissue, but did not carry the cultures through a series of transfers. Successful infection was achieved by inoculating spores from two-membered cultures on seedlings. Establishment of the mildew occurred only on tumor tissue and not on callus tissue, but only two of about one hundred trials were successful. The successes reported were with tumor tissue which was growing rapidly, in agreement with evidence that active growth of the host favors these parasites.

In 1901 Ray (110) reported casually, but without data or supporting evidence, the saprophytic cultivation of rose rust and *Euonymus* rust on a gelatine medium containing plant extracts and spoke of the teliospores of the former being produced within the gelatin medium. Such cultures of parasitic fungi, he said, were less virulent than cultures on the host. In 1936, Gretchushnikov reported the saprophytic cultivation of species of *Puccinia*, which were kept growing for twelve days on a culture medium containing substances which adsorbed ammonia and urea produced by the growing mycelium (111). These compounds had been found to inhibit development of rust spores, and susceptibility was considered to depend upon their continuous removal by the host plant. In 1951 Hotson & Cutter reported the cultivation of *Gymnosporangium juniperi-virginianae* on a relatively simple artificial medium containing mineral salts, a carbon source, ascorbic acid, and yeast extract (107). Subsequently, Cutter reported that biotin was the only growth

factor required, and that additional saprophytic cultures had been obtained (73). These cultures arose by growth of mycelium out of callus tissue cultures of the rust after several months incubation, and were reported to reinfect sterile tissue cultures of the alternate host (*Crataegus*). The emergence of a saprophytic mycelium was extremely rare, a fact which led Hotson & Cutter to suggest that the saprophytic growth arose by mutation from the parasitic mycelium. In a later paper reporting the behavior of this same rust on tissue cultures, Hotson makes no mention of the occurrence of saprophytic growth, and does not refer to the earlier paper on which he was senior author (108).

These reports leave no doubt that the cultivation of obligate parasites on undifferentiated tissues of higher plants is now feasible, but the answer to questions concerning the nature, origin, and methods of maintaining saprophytic cultures of rusts awaits further work.

Alterations in Host Composition and Metabolism

The effects of a fungus growing on a higher plant are referred to as the symptoms of disease when the effects are judged to be generally deleterious, and the fungus is then considered to be a parasite. The definition of parasite is thus an arbitrary one. The effects of obligate parasites in the earlier phases of their development are frequently not detrimental to the susceptible host, and may in fact be regarded as beneficial, at least to some of the host tissues. When developing on a susceptible host they are well along the path toward that complete compatibility which is the goal of perfect parasitism (112) and which, when reached, is regarded as symbiosis.

Although the initial response of a plant to infection is induced by the invading parasite, the following events are the result of interaction between the parasite and its host. Just as changes in the external environment may drastically alter resistance or susceptibility, so may internal changes induced by the parasite influence its own development and the trend of the subsequent association. The cause of changes, particularly those which arise in the later phases of disease and are therefore the resultant of a series of events is difficult to assess with any confidence, and there is very little information in the literature which can help 'n this assessment. Whether the changes observed at any one moment in the disease originate in the host cells or from the fungus itself has often not been determined with certainty, except for processes like photosynthesis to which the fungus does not contribute. Diseases caused by powdery mildews are particularly suitable for making this distinction (113) since, except for haustoria, the parasite mycelium is entirely outside of the host tissues and may be separated from them at any time. With other obligate parasites mycelium-free tissues near the site of infection may be separated from infected tissues either visually or by actual operation. Other possible techniques for distinguishing the host activity from that of the parasite, such as microscopic observations, have been applied to some extent.

The role of toxins.—The role of toxins produced by the parasite in bringing about the symptoms of disease has been well established for many facultative parasites and deduced for obligate parasites on the basis of less direct evidence. The violent response to initial penetration in resistant plants and the response of susceptible tissues free of fungus mycelium constitute convincing evidence for the participation of toxins. Responses in healthy plants have been induced by extracts of diseased tissue (92, 111, 114, 115), but the substances responsible may be either of fungal or host origin.

The substances initially introduced and originating from the parasite occupy a key position in problems of resistance and susceptibility. Such substances must be postulated when a host cell responds to the presence of hyphae or the entry of a haustorium, as in a wheat plant showing a hypersensitive reaction. If that same parasite fails to produce the response in another wheat plant whose cells it also enters (a susceptible genotype), the substances concerned must be either (*a*) without effect upon the second cell, or (*b*) neutralized by the second cell, or (*c*) prevented from appearing in the second cell. Each of these alternatives, but particularly the second, has been more or less explicitly proposed (19, 20, 43, 94). To distinguish amongst them might be possible if a preparation having activity against the resistant host could be obtained. Since the responses involved are elicited by the germling, an approach to the question need not await the cultivation of rusts or mildews on an artificial medium, but might be made with germinated spores as a source of activity.

Changes in permeability.—Amongst the earliest results of invasion by rusts and mildews are changes in the concentration and distribution of solutes. Decreases in osmotic pressure and increases in permeability were reported by Thatcher for susceptible hosts (see above for method), whereas permeability decreased in resistant hosts (92, 93). The increases in permeability were greatest in cells adajcent to rust colonies. He suggested that the resulting loss of solutes played an important part in making nutrients available to the parasite. On wheat plants with a mesothetic reaction, decreases in permeability were associated with the resistant type colonies, increases with the susceptible type (94). An increase in permeability following infection of susceptible hosts by rusts was inferred by Humphrey & Dufrenoy (95) from observations on stained tissue sections, which showed intercellular accumulation of solutes, notably soluble phosphorus compounds. This accumulation was reported to be not only at the expense of adjacent cells but also of tissues at some distance from the infection court. Permeability increases have also been reported for several other rust and mildew infections (9).

The accumulation of substances in infected tissues (116) appears to depend not only upon retention of the products of photosynthesis but upon an actual mobilization of materials from the healthy tissues of the plant (117). The metabolic dependence of solute transport in other biological systems suggests that this mobilization of substances in infected tissues probably has its origin in metabolic changes and not simply in the altered structure of a

putative protoplasmic membrane across which solutes move in obedience to the laws of diffusion. To detect the ultimate metabolic basis of altered solute distribution would require more refined techniques than those employed in the above studies.

Changes in nitrogenous compounds.—Although several investigators have reported accumulation of nitrogenous materials following infection, particularly by rusts, there are differences of opinion concerning the type of nitrogen compound accumulating. Daly (55) has reported some data which indicate that the accumulation in wheat leaves of soluble nitrogen compounds (ammonia, amino, and amid nitrogen) but not of protein is greater after infection when the host resistance is increased by supplying ammonium nitrogen instead of nitrate. Gretchushnikov found relatively large amounts of ammonia and urea in dried rusted leaves, and apparently associated them with resistance (111). More recently he has reported a general increase in degradation processes in potatoes infected with *Synchytrium* (114). There are similar reports for several hosts infected with rusts and powdery mildews (9). Gassner & Franke's extensive analysis (118) indicated that nitrogen was retained in wheat infected with leaf rust, while disappearing in uninfected leaves. Both protein and soluble nitrogen compounds persisted, but soluble nitrogen showed a relative and sometimes absolute increase. D'Oliveira (119) recorded a net increase in the total nitrogen content of rusted wheat and barley, after the seedlings were cut off and kept in distilled water for twelve days. He suggested that the increase resulted from nitrogen fixation, but the variability between duplicates suggests that the differences observed may not have been significant. Chlorophyll retention in the tissues surrounding a colony is a very common phenomenon not only in leaves infected with obligate parasites but also with some facultative parasites, and is particularly conspicuous on leaves placed in the dark after the parasite is established. Allen (117) reported that the tissues of wheat adjacent to powdery mildew colonies first lose their chlorophyll and then regenerate it. Determination of total chlorophyll showed a general decline commencing after two or three days, with some indication of a net increase in the later stages of disease. Loss of chlorophyll is not one of the first symptoms of disease but occurs in later stages of shoot or systemic infections caused by many different obligate parasites (9, 41).

Amylase in wheat infected with powdery mildew (65) and with rust (7), lipase in wheat infected with mildew (65), peroxidase in potato tubers infected with *Synchytrium endobioticum* (120) and in rusted wheat (7), and catalase in lupine infected with powdery mildew (9) have all been reported to increase. Later decreases in enzyme activity were found in nearly all of these investigations. Kuprevicz found only decreases in catalase activity in several rust infections (9), although he has reported high catalase activity in germinating rust spores (121).

Changes in lipids and carotenoids.—A striking accumulation of lipoidal materials at the site of infection was observed by Schmidt as an immediate

response to invasion of sugar beet leaves by *Uromyces betae* (122). Similar appearance of lipoid globules occurs in wheat infected with stem rust and is particularly conspicuous in leaves showing the browning reaction (94). Increases in β-carotene and the appearance of considerable amounts of γ-carotene, which does not occur in detectable amounts in healthy leaves, have been found in crab apple leaves infected with the pycnidial stage of *Gymnosporangium* (123). The galls of the telial stage in juniper are also enriched in γ-carotene (124). Carotenoids are present in wheat seed from rusted plants in sufficient amounts to color the flour (125), suggesting that formation of carotene is induced in host tissues. Singalovsky reported loss of carotenoids from sugar beet leaves infected with *Peronospora schactii* (41).

Changes in growth factors and auxins.—Increases in pantothenate content, up to 30 μg. per gm. dry weight (compared with about 2 μg. in healthy leaves) were found by Yarwood, Hall & Nelson (126) ten to twelve days after inoculation of Pinto beans with *Uromyces phaseoli*. Rust spores are also rich in pantothenate (45 μg. per gm.) but the increase in rusted leaves is too much to be accounted for by pantothenate in the rust alone. Rusted leaves also produced greater growth responses than healthy leaves when fed to snails. The quantitative assays were made with *Lactobacillus* and do not therefore include the pantothenate bound in coenzyme A (127).

Many of the responses of higher plants to foreign organisms indicate changes in the content or distribution of auxins. Indoleacetic acid is a common if not universal product of fungus metabolism, though some fungi are inhibited by such low concentrations that auxin has been suggested to play a part in host resistance (128). Studies with facultative parasites have revealed correlations between auxin production by the fungus in culture, auxin content of infected tissues, and the type of symptoms (129, 130). Slankis has shown that the dichotomously branched short roots typical of some conifers with mycorrhizal infections may be induced in roots by the culture filtrate of the mycorrhizal fungus and by either indoleacetic acid or naphthaleneacetic acid at concentrations of 0.5 to 10.0 p.p.m. (131, 132, 133). Systemic infections with rusts generally result in gall formation and witches brooms or distorted growth. Abnormalities of growth reminiscent of the effects of some auxin analogs are often produced by downy and powdery mildews. Premature leaf abscission in beans infected with *Erysiphe polygoni* occurs while the leaves are still green suggesting a decrease in auxin (134). Pilet has investigated the auxin content of *Sempervirens tectorum* leaves infected with the rust *Endophyllum sempervivi* (135). Infected leaves are about twice as long as healthy ones, narrower at the base and light green in color. A maximum concentration of $10^{-7} M$ was found near the middle of healthy leaves, whereas a maximum of $10^{-4} M$ occurred near the locus of infection in diseased leaves. In other regions of the leaf even a centimeter or more from the edges of the fungus mycelium, the auxin content was higher than in comparable regions of healthy leaves. Pilet's method of auxin determination (136) involved extraction with chloroform and assay by the *Avena* curvature test. There is not

conclusive evidence as to whether the extra auxin originated from the rust or from the host cells. Abnormally high auxin concentrations in *Euphorbia cyparissias* infected with *Uromyces pisi* were also indicated by Pilet (137). The stems of lightly infected plants were still positively phototropic, but if heavily infected they became negatively phototropic. Addition of $10^{-5}M$ indoleacetic acid to the culture solutions of infected plants caused the stems to become positively geotropic but did not have this effect upon healthy plants. Whereas light- and gravity-induced increases in auxins stimulate growth in normal plants, in the rusted *Euphorbia* the increases lead to supra-optimal concentrations and result in reverse tropisms, a result which would be expected if the initial concentration is already high in the diseased plants.

The similarity of a response to the effects of auxin does not necessarily signify an increased auxin content, since the same response may be elicited either by increases in auxin or by reduced amounts of other substances such as adenine (138). Furthermore, other substances affecting growth besides indoleacetic acid have been reported from diseased plants. A volatile material assayed by its inhibiting action on pea seedlings and presumed to be ethylene, was found by Williamson (139) to be produced in greater amounts by rusted leaves of snapdragon and chrysanthemum than by healthy leaves of these plants. Rusted leaves had less activity, however, than injured leaves or leaves infected with facultative parasites, and mildewed rose leaves were inactive.

Accumulation of carbohydrates and phosphorus.—Although decreases in carbohydrate content have been reported in some instances (40, 41, 120), increases appear to be characteristic of the early phases of infection in susceptible plants with good illumination. Although little or no starch is formed in healthy wheat leaves, it accumulates around colonies of the parasite when wheat is infected with leaf rust (140) or powdery mildew (117) and there are marked increases in the total starch, reducing sugars, and sucrose during the first week following infection with mildew. Kuprevicz (9) reported less total carbohydrate accumulation during the day in several rusted plants than in healthy, but noted a preferential accumulation of monosaccharides in rusted thistle plants. Conversion of starch to glucose and accumulation of the latter occurs in potato warts (141).

The carbohydrate accumulation in mildewed wheat is not accompanied by a net increase in soluble organic phosphates (142) but phosphorus in some form is definitely accumulated in the region of rust colonies. This has been shown clearly by Gottlieb & Garner in wheat leaves infected with *Puccinia graminis tritici* (143). The first foliage leaf of seedlings was infected, and all other leaves removed as soon as they appeared. Radioactive phosphate was then supplied to healthy and infected seedlings, and shown by radioautographs to accumulate around each rust pustule. Since the specific activity of spores from these pustules was less than that of either healthy or infected leaves, it is unlikely that the extra phosphorus was entirely in the rust plant, although this possibility could not be conclusively rejected. No

significant increase in the total phosphorus of infected over healthy leaves
could be detected. These findings confirm other indications of phosphorus
mobilization in the infection court (95).

Changes in photosynthesis.—Increases in photosynthesis during the first
few days after infection of bean and wheat leaves with powdery mildew have
been reported (80, 134, 144) but are not always evident (9, 117). Within a
few days of infection, definite decreases occur, commencing sooner with
heavily infected than with lightly infected plants, and continuing while the
carbohydrate content is still increasing (117). In the earlier literature, de-
creases in photosynthesis were reported for several other rust and mildew
diseases.

Increases in respiration.—Augmented respiration is almost a universal
consequence of the infection of higher plants with obligate parasites. The
literature on this subject has been summarized in recent reviews (9, 80, 142).
Much of the increased respiration is contributed by the host cells (113, 145)
and probably involves oxidation of carbohydrate during the period of vegeta-
tive growth of the parasite, since the respiratory quotient is close to 1.0 until
the rate of respiration and the growth of the parasite begin to decline (117).
Smaller increases (113) or actual decreases (80, 144) in anaerobic carbon
dioxide production accompany the increased respiration, and it is clear from
Sempio's data (144) that these changes are accompanied by an inhibition of
the Pasteur effect. The augmented respiration in mildewed wheat leaves is a
joint result of an increase in soluble carbohydrate and removal of the aerobic
inhibition of carbohydrate breakdown. If removal of the aerobic inhibition is
effected by an uncoupling action similar to that of 2,4-dinitrophenol, the
resulting increase in intermediates would be confined to degradative proc-
esses and would be achieved by rapid regeneration of phosphate acceptors
and of inorganic phosphate with loss of energy rich phosphate. From the
many indications that synthetic processes are also accelerated in plants in-
fected with obligate parasites, it would seem that an uncoupling action is not
the primary or exclusive cause of augmented respiration. The increase of
phosphorus in the infection court, the stimulation of host cell growth by many
obligate parasites and of synthesis of various substances by others, are evi-
dence for an increase in the utilization of energy-rich phosphate as the cause
of increased respiration. Greater hexokinase activity could have this effect
(146), as it does in many systems, including mitochondrial preparations from
plants (147), and would lead to increased formation of glucose-6-phosphate,
part of which might be diverted to starch synthesis. Opening other channels
of utilization of energy rich phosphate would also permit a more rapid aero-
bic breakdown of carbohydrate, accompanied in this case by an increased
production of intermediates along another synthetic path. Sempio considers
that the changes in host metabolism after infection as well as those induced
before infection, favor the further development of the parasite, and Cutter
has recently outlined evidence in support of the view that phosphorylated
intermediates of host metabolism are of critical importance in the develop-

ment of obligate parasites (73). There are many signs which point in this direction, but whether the crucial substances lie along the path of carbohydrate breakdown or on the route of synthesis has not yet been clearly indicated. Since it is possible to block one path without simultaneously eliminating the other, this question could be put to experimental test.

Effect on virus multiplication.—Besides the favorable effects upon development of other obligate parasites, rusts also produce changes in the host which markedly accentuate the infectivity, multiplication, and invasiveness of viruses (102, 103). Bean plants otherwise resistant to tobacco mosaic virus became susceptible if first infected with *Uromyces phaseoli*. In susceptible leaves, progressive spread of tobacco mosaic virus occurred instead of the usual local necrotic lesions. Several-fold increases in the number of infective particles were produced by twenty minutes' incubation of tobacco mosaic virus or tobacco necrosis virus with extracts of rusted plants, whereas incubation with healthy leaf extracts had only a slight effect upon multiplication. Effects upon other viruses and by other rusts were also found. These reports are of particular interest in view of the generally inhibitory action upon these viruses of filtrates from cultures of saprophytic fungi (148). At least part of this effect may be due to calcium pantothenate, which also increases the infectivity of a virus preparation and is present in much greater concentration in rusted than in healthy leaves (126).

Conclusion.—The two central problems concerning the parasitism of rusts and mildews—the mechanism of resistance and susceptibility and the nutrient contributions of the host—are still far from solution. Nevertheless, the investigations to date have given satisfactory answers to some ancillary problems and have revealed some lines of approach to the major problems which seem quite promising. There seems little doubt that the study of the physiological bases of resistance and susceptibility will benefit greatly from a rigid control of the genotype of both host and parasite. The substances involved in the initial determination of resistance and susceptibility are probably distinct from the nutrients required to support the vegetative growth of the parasite. Interpretation of results in many kinds of investigation would be greatly facilitated by adoption of criteria for susceptibility which make a clear distinction between differences due to number of spores reaching the host, numbers germinating, numbers infecting, and the rate and amount of subsequent development.

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THE ROLE OF PLANT PHYSIOLOGY IN PLANT GEOGRAPHY[1]

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INTRODUCTION

Plant geography embraces all aspects of the science of comparative floristics. Its recognizable facts are the results of causes that have operated in the remote and immediate past. Therefore, we may seek explanations for plant geography on planes similar to those employed by the paleontologist, in that we use knowledge of plants as they operate today as clues for understanding their operation in the past.

In this review we are concerned with the contributions of plant physiology to interpretations of plant geography. Presently known principles of plant physiology provide a guide to theory and a framework for logic. Also, the research methods of plant physiology may be brought to bear on the question of how the dynamics of plant geography have been initiated and how they have developed toward results now revealed from studies of comparative floristics. Such researches have been of two kinds, each worthy of separate consideration. First, there are the experiments designed to test hypotheses which have been presumed to explain effectively an observed fact in geobotany. Secondly, there are researches into the genetics of preadaptation which are very important to the problems of speciation and migration as these affect elaboration over area. These genetic researches pertain to the inheritance of characters controlling the physiology of environmental relations. They have included special problems on what have been termed "ecotypes" and "ecoclines" wherein knowledge has been sought concerning the mechanisms responsible for environmental selection.

Although the scope of researches just outlined may seem very broad, unhappily, the large body of literature of plant geography only rarely reports the conscious applications of plant physiological research as a means of arriving at conclusions. This, it seems to us, has resulted from three principal causes. First, much of the literature of plant geography has been contributed by authors who have not had special training in the principles of plant physiology, or, at the time of writing, the principles then recognized had not advanced adequately to be of use in the problems discussed. (This latter aspect plagues everyone in interpretive science.) Secondly, the plant physiologist has been reluctant to carry his studies of plants to their natural environments because difficulties of establishing controls do not permit adequate testing of the validity of the conclusions reached in the laboratory. However, much truth is discoverable from observational studies of a physi-

[1] The survey of the literature pertaining to this review was completed in February, 1954.

ological character, and from them hypotheses for more critical testing are possible. Unfortunately, most of the nutritional experience has been gained from agricultural rather than from wild plants. Agricultural plants are often relatively pure lines genetically, while the populations of wild plants to which the tests were being applied are more often heterozygous. Obviously, if adaptability of strains is important for survival of the species, a pure genetic line is the wrong kind of control plant. A third difficulty stems from the complexity of environmental relations, and from the impediments to be met in isolating tolerances for study. However, some progress has been made in the field of population genetics and some of the basic patterns of reactions have been disclosed.

The literature pertaining to presumptive cause and effect relationships in plant geography is truly enormous. In order to confine our task to a workable area we have adopted as basic, the philosophy that all conditions or events affecting the geography of plants exercise their influence on plants as individuals. This will free us from considering the tremendous numbers of works wherein conclusions have been based upon presumed functioning of aggregate classificatory groups such as plant communities, floras, or other of the hierarchy of taxonomic or vegetational categories into which groups of individuals may be mentally classified, and which are so often employed as the unit of discussion in the problems of plant geography. It is our contention that if the ideas of plant physiology are to have significance for plant geography, they will have to be considered in terms of physiological processes functioning in individuals. Although it is possible to conceive of an aggregate effect of individuals for purposes of classification, such aggregate effects are conceptual only and, therefore, are abstract.

If one were to make an exception to the above remarks, it would have to do with the interbreeding population. Although the cytological and physiological aspects of genetics operate strictly on the functional level of the individual organism, the statistical aspect of the interbreeding population operates as a reservoir of variables, making it desirable to stress this role of interbreeding in our problem. It is impractical to attempt to separate the roles of genetics and physiology in plant geography. The chronology of the events affecting plant geography demands reproductive successions of individuals. Such reproductive succession sets in motion the mechanics of population genetics which become significant to our problem since they are concerned with preadaptation and environmental selection resulting in alteration of floristics. Mason (28) has suggested that the forces of evolution and those of elaboration of plants over area are alike and concomitant.

The hereditary nature of physiological characters of plants is no longer just an assumption. The work of Turesson (43) and of many subsequent workers has made this amply clear. Since most of the significant aspects of our problem are concerned with preadaptation of seeds to conditions in which they may function as individuals, and the selection of these individuals by the environment, we will not review earlier work than the 1922 paper of

Turesson on the "Genotypical Response of the Plant Species to its Habitat," except where it seems desirable to use background material.

Our problem, therefore, resolves itself into three phases. First, we shall deal with the development of principles by the physiologist and the conscious application of these principles to the problems of plant geography. Second, we shall consider researches revealing the genetics of habitat relations as they pertain to the physiological capacity of individuals, to preadaptation, and to the general elaboration of populations over area as these facts have been arrived at experimentally. Finally, we shall deal with those few works that are concerned strictly with experiments which reveal the physiology of floristics.

APPLICATION OF PHYSIOLOGICAL PRINCIPLES TO PLANT GEOGRAPHY

Aside from the general principles of plant physiology as they should serve to condition theory and logic in the interpretation of the problems of plant geography, there are certain principles which have been directly taken over and stated, or restated, in terms of their role in plant geography. These pertain primarily to the limiting factors of the environment and to the tolerances of plants.

There have been several attempts to state the laws of plant geography in terms of generalizations [Cain (7); Good (12, 13); Mason (24)]. Each of these workers, in turn, has considered those generalizations which pertain to such matters as tolerance by the plant of environmental condition, and to the concept of limiting factors. Each has presented something that is a modification of Liebig's (21) law of the minimum or of its subsequent restatements by Blackman (6), Lundegårdh (23), and Taylor (39), or of the principle of limiting factors as stated by Livingston & Shreve (22). Prior to Livingston and Shreve the concept was expressed largely in terms of the role of environmental factors which were close to the minimum level.

The law of the minimum as outlined by Liebig was thought of as applying to nutrients present in soil solutions. Briefly stated, it maintained that, "The yield of any crop always depends on that nutritive constituent that is present in the smallest amount." The emphasis here is upon nutrient constituents or substances in solution. Obviously, although the major point is pertinent, there are many aspects of limiting factors that are not included in Liebig's law as stated. Blackman (6) attempted a revision to broaden its scope. Although Blackman's statement has been referred to as the law of limiting factors it still emphasizes factors in the minimum and must be regarded as a modification of the law of the minimum. Blackman stated, "When a process is conditioned as to its rapidity by a number of separate factors, the rate of the process is limited by the pace of the slowest factor." Here, obviously, there is no discrimination as to the kind of factors. Lundegårdh (23) thought that neither of these statements adequately incorporated the interaction of factors and he proposed a statement which included the

relative effect of the factor as it approached the minimum. It is this restatement that is of special interest to the plant geographer. Lundegårdh stated that,

> The more nearly a factor is in the minimum in relation to other factors acting upon the organism the greater is the relative influence of a change of that factor on the growth of the organism. As a factor increases in intensity its relative effect upon the organism decreases; and when the factor is in the region of the maximum the relative effect upon the organism is nil.

Lundegårdh's statement is known as the law of relativity.

Another revision of Liebig's law was made by Taylor (39) who was concerned with abnormally cold weather in its affect on populations of animals. Taylor was thinking of the problem solely from the point of view of the law of the minimum of Liebig and apparently not in terms of the relative effects pointed out by Lundegårdh. He was impressed by the necessity of thinking of the problems in terms of the condition of the organism at the time of the application of the factor, which he represents in terms of the most critical time of the year. The assumption is that at this time of year the organism is always vulnerable. He also was impressed with certain cyclic phenomena of animals presumed to be related to climatic cycles, and he included this in his statement. His rewording of the law of the minimum states that, "The growth and functioning of an organism is dependent on the amount of the essential environmental factor presented to it in minimal quantity during the most critical season of the year, or during the most critical year or years of a climatic cycle." Taylor's concept could be thought of also in terms of stages of ontogeny of the organism which would be stopped or retarded by factors in the minimum. That this also applies to plants is borne out by common observations during the same cold winter of 1932 in which Taylor observed the animal populations in Arizona. In California plants growing under the protection of lath were killed while the same species in the open survived. The conditions of the protection resulted in tissues which were especially vulnerable to freezing as compared to plants grown in the open. From the point of view of laws of the minimum these generalizations are important for interpretations of plant geography because they provide a basis for explaining floristic diversity through habitat diversity. For instance, Mason (27) has pointed out that the great ecological and floristic diversity of the southwestern United States is explicable in terms of the superposition of high relief and its attendant variation in edaphic mosaic over a geographic moisture gradient that approaches the minimum. Under these conditions, with respect to the factor in the minimum, namely moisture, small differences have great local significance. In an arid region such as that in and surrounding a desert, an additional two inches of rainfall a year would be very significant and might, in some places, double the seasonal quota. On the other hand, in a region of high rainfall an additional two inches would be

negligible in its effect. These situations explain the great geographic diversity of floras in temperate arid regions as contrasted with the greater uniformity of floras over a temperate humid region. In arid regions the diversity in the habitat is intensified, creating a condition of high selective potential. Such regions are characterized by many polymorphic genera and species. This is an area of science where every problem is a problem in the genetics of natural selection as it pertains to the physiology of habitat relations.

Livingston & Shreve (22) thought of limiting factors not only in terms of the minimum but in terms of their limits as expressable in terms of extremes. In their theory of physiological limits they state, "For every vital function there is a maximum and a minimum zero point with respect to any conditioning factor beyond which functioning ceases." They were concerned here primarily with problems of the distribution of plants, stating that, "It is mainly in accord with the generalizations of the theory of physiological limits that distinct climatic areas are characterized by corresponding types of vegetation, and the principle is probably of primary importance in the study of plant distribution." They also point out the difficulties which arise in applying such a principle to precise problems because of the collective nature of the action of environmental influences. They state, "In an investigation of the role that is played by the various climatic conditions in determining the optimum activity of the plant or the limitation of its distribution, it is necessary to bear in mind that the conditions act collectively and that their influences are interdependent. The role of each condition changes with the changed values of other conditions. In attempting to determine the relative importance of several climatic conditions as determinants of a given distributional phenomenon, it is seldom possible to do more than speak in general terms. It may be possible to state, for example, that temperature conditions are more important than moisture conditions in a given case, without its being possible to determine, on the same evidence, which of the various aspects of temperature is most important." They point out further that these problems are essentially physiological in their scope since they rest "upon the influence exerted by environmental conditions on the activities of individual plants."

A further contribution to these concepts was presented by Good (12) in his theory of tolerance. Whereas Livingston and Shreve thought of the problems in terms of limits within the plants to environmental factors, Good thought of them in terms of a span in which it is possible for the function to operate. The differences appear to be subtle, but the concept of limits is perhaps more in line with the problems of plant geography since we are especially concerned with phenomena that fix the periphery of area. Here at the periphery we are less concerned with the interaction of factors, for the precise delimitation may be, and often is, a single factor in a given segment of the periphery of area. This, it must be pointed out, does not mean that the entire periphery is under the regime of the same factor, nor does it mean that

the functioning of the plant near the periphery of area is less complicated in its relation to the environment than it is in the interior of its area. Good worded his theory of tolerance as follows:

Each and every plant species is able to exist and reproduce successfully only within a definite range of climatic and edaphic conditions. This range represents the tolerance of the species to external conditions.

The tolerance of any species is a specific character subject to the laws and processes of organic evolution in the same way as morphological characters, but the two are not necessarily linked.

Change in tolerance may or may not be accompanied by morphological change, and morphological change may or may not be accompanied by change of tolerance.

Morphologically similar species may show wide differences in tolerance and species of similar tolerance may show very little morphological similarity.

The relative distribution of species with similar ranges of tolerance is finally determined by the result of competition between them.

The tolerance of any larger taxonomic group is the sum of the tolerances of its constituent species.

The chief merit of the theory of tolerance as propounded by Good rests in calling attention to the foundation of environmental relations in genetics, and through this, to point out that these relations may be expected to be as variable as are morphological characters although the two are not necessarily linked. It is also interesting that Good should call attention to the fact that tolerances are specific characters. However, if this fact is to be useful it can only serve those purposes of logic to which an abstract fact may be put. Perhaps its greatest importance rests in the significance of its place in the interpretations of the enveronmental relations within populations, and as a guide to the comparison of different populations of the same species where it serves to remind one of the reservoir of variability which occurs in the species as a whole.

Since tolerance of a species is an abstraction, the tolerance of any higher category can only be the compounding of an abstraction at which Good would arrive through a summation. We doubt if this serves any useful purpose even in logic. Since tolerance is significant only to an individual, it is doubtful if even a useful typology would result from the summation of the tolerances of a higher category of taxonomic unities. No family is ever characterized by the sum of the characters of its constituents. Only the common characters are utilized for such abstraction. A summarization of the common tolerances might serve a useful purpose to a problem in plant geography. For instance, the tolerance of submerged aquatic conditions characterizes the entire Potamogetonaceae. There are differences in tolerances to other environmental conditions which account for the special geographic problems within the family. If all families have common distinctive tolerances, these tolerances have not as yet been discovered.

The trouble seems to rest in the aggregating of tolerances into an abstract total unitary concept, whereas they are only useful in their separate func-

tion-factor relationships of individuals. Even the submerged condition of the members of the Potamogetonaceae is meaningful only in terms of the plant functions that are affected. Such relations within the group are comparable only to the extent that they involve comparable functions in different plants.

With respect to Good's contention that "the relative distribution of species with similar ranges of tolerance is finally determined by the result of the competition between them," we can comment only that the general usage of the concept "competition" with respect to plants is so all-embracing that it becomes meaningless. It would be a long step toward clearer expression and greater precision of language if we would refrain from referring cases to competition in which inhibition has not been demonstrated.

Good (13) incorporated his theory of tolerance in his "The Geography of the Flowering Plants" with no significant additions, merely summarizing what he had said originally. In addition to the theory of tolerance, he treated, in both of his works, six other principles of plant geography as follows:

Plant distribution is primarily controlled by the distribution of climatic conditions.
Plant distribution is secondarily controlled by the distribution of edaphic conditions.
Great movements of species and of floras have taken place in the past and are apparently still continuing.
Plant movement, especially in the larger aspect of plant migration is brought about by the transport of individual plants during their dispersal phases.
There has been great variation and oscillation in climate, especially in the higher latitudes, since angiosperms became prominent.
At least some and probably considerable variation has occurred during the same period in the relative distribution and outline of land and sea.

Mason (24), after consideration of problems in the fossil record, accepted Good's major thesis and also, with some minor alterations, accepted the principles presented by Good as well as adding others which he deemed important. The first of these minor variations was the addition to Good's first principle, of the concept that the means of climatic factors in any given region were less significant than the extremes. This concept resulted from a consideration of the causes involved in the periphery of area which are so important to plant geography. These are all problems of the extremes of environmental condition. On the other hand, the mean is an abstraction for human usage and is of no significance to the plant. This change was added solely because the mean is currently used so often in a role of assumed significance in cause-and-effect relationships.

Another modification, relating to Good's fourth principle, added the concept of establishment to that of dispersal since migration is not effective without establishment. With respect to physiological matters, considerable change in the wording of the theory of tolerance and the concept of limiting factors was expressed in the following two statements:

The functions governing the existence and successful reproduction of plant species are limited by definite ranges of intensity of particular climatic, edaphic, and biotic factors. These ranges represent the tolerance of the function for the particular factor.

In the life history of the organism there are times when it is in some critical phase of its development which has a narrow tolerance range for a particular factor of the environment. The distribution of this intensity span of the factor during the time the plant is in this particular phase limits the area in which the function can operate, and hence governs the distribution of the species. The narrower the range of tolerance, the more critical the factor becomes.

These two statements call attention to the necessity of considering tolerances in terms of precise factor-function relationships since an abstract statement relative to over-all or to group tolerances cannot be significantly phrased in terms that are objectively applicable. Furthermore, there is an attempt to bring in the concept of the critical ontogenetic phase of the organism as expressed by Taylor (39) in his restatement of Liebig's law. It also lays a foundation for a concept of the range of tolerance of an individual and possibly for a population or for a species, as being the range between the most critical or highest minimum of any factor-function relationship and the most critical or lowest maximum factor-function relationship. The factor-function relationships in each extreme are not necessarily the same.

Mason also pointed to the need for considering the problem of perpetuation and evolution of floras since this plays an important role in the interpretation of plant geography utilizing the facts from the fossil record. Two statements follow:

"The perpetuation of vegetation is dependent first upon the ability of species to migrate, and secondly upon the ability of species to vary and transmit favorable variations to their offspring."

"The evolution of floras is dependent upon plant migration, the evolution of species, and the selective influences of climatic change acting upon the varying tolerances of the component species."

These statements were made in an attempt to point out that the persistence of vegetation on the earth was both orderly and dynamic and that both persistence and change involved the processes of genetics and migration. After considering the problems of evolution and migration, Mason (28) concluded that these were concomitant phenomena resulting from precisely the same causal dynamics.

When considering the problems of the history of the genus *Ceanothus*, Mason (25) stated, "It can be taken as axiomatic in plant geography that the capacity of the plant species to tolerate or to respond to its environment is governed by the laws of evolution and genetics, and the range of tolerance is the direct result of the genetic diversity of the species: that is, the mutation, combination, and recombination of the genetic units within the species are responsible for variations in tolerance upon which the environment acts in the selection of its biota." This was recognized but covered in less detail by Good in his theory of tolerance.

Cain (7) accepted the principles of plant geography as laid down by Good and by Mason, but expressed them in many fewer words, and added two further points. The first of these two points is self-evident, namely, that biotic factors are also important in controlling plant distribution. We, however, would urge caution in applying this concept because it demands careful scrutiny in determining if there is clear evidence of a causal relation with the biota. Furthermore, it demands discrimination between direct and indirect relationships. Because of the difficulty of interpreting indirect relationships, many of them are best handled strictly on a plane of environmental factors of a physical nature without implication as to their cause. The reacting plant, so far as we know, is unable to discriminate between sources of environmental conditions. Many of the fantasies that have entered the literature of plant geography and ecology stem from a lack of caution and clear discrimination in problems that are presumed to be based upon biotic factors.

Cain's second contribution states simply that the environment is holocoenotic, meaning that the plant as a functioning organism is conditioned simultaneously and collectively by all of the factors of the environment. This is a difficult concept to assess both in terms of its truth and in terms of its role in plant geography. In the first place, Cain goes further in his statement and interpretation of holocoenotic environments than do Allee & Park (1) in their original statement of the concept. These authors made no claim for an environment that is both "collective and simultaneous" in its action, but thought of it as an interacting and interweaving system. Nor do these authors assume that a holocoenotic environment precludes a factor operating in its individual capacity. Quite the contrary, they point out that single factors may operate independently and in this capacity be limiting. Also they allow for a possible analysis of the system. [For a further discussion see Billings (5).] We are therefore concerned here only with the concept of holocoenotic environments as developed by Cain.

It seems to us that a concept of a collective simultaneous environment over any extended period of time does violence to those very environmental relations which caused Cain to include as a "law" of plant geography that, "Different ontogenetic phases have different tolerances." Our objection would be overcome if the concept of holocoenotic environments were prefixed by the phrase "At any given time." This, however, would reduce its force and make of it a self-evident truth scarcely deserving of more than the casual mention that it received originally from Allee & Park (1). To reduce it to less abstract language one would have to state it thusly: at any given time, all of the active factor-function relationships of a plant operate together, and (according to Allee and Park) may be interrelated through their effect upon one another.

EXPERIMENTAL STUDIES

There has been very little in the history of plant geography that can be classified as an experimental study and far less that can be regarded as a

physiological experiment. Certain early studies are classic. These, aimed to determine whether or not the concepts of dispersal by ocean currents or dispersal by birds, constituted a possible and effective explanation of these problems, particularly as they pertained to the floristics of oceanic islands. Darwin (11) soaked seeds of many kinds of plants in salt water to determine the length of time they would remain viable. He then correlated his findings with the speed of certain ocean currents and concluded that under favorable conditions those seeds which withstood salt water longest would be able to travel about 900 miles at the most before they would perish. Kerner (18) fed many kinds of seeds to several different kinds of birds to determine their viability after they were passed through the intestinal tract. He correlated his findings with the length of time required for the seed to pass through the intestinal tract, and concluded that this was so short that the bird would not be able to travel very far before the seed was eliminated. There were several such studies by different workers. We point out these as background to indicate the nature and scope of some of the early studies.

Although many concepts of plant geography pertaining to the occurrence of plants in special habitats rest upon conclusions of the plant physiologist, it is interesting to find that most of our information on this subject is purely observational and not experimental. Plant geographers have developed concepts concerning plants found on special soils which are related, in turn, to their parent rocks. However, very little research has been directed toward determining why the components of the native flora differ in their ability to exist on soils of different origin. As an example of this, the serpentine problem has attracted the greatest amount of attention. Early research on serpentine soils in relation to plant nutrition has been summarized by Robinson, Edgington & Byers (31) and more recently by Rune (34). Other problems of endemism [Mason (26)] are largely concerned with special habitats involving volcanics, granodiorites, and other igneous rocks, as well as gypsum and dolomites. In addition, there are the endemics found in alkaline sinks and in vernal pools in arid regions.

It was not until the researches of Turesson (43, 44, 45) that the problems of the environmental relations of plants in their bearing upon the geography of plants began to be clarified. With his work the concept of natural selection began to make sense and to fit in with the findings of the geneticist. It marks the beginning of the neo-Darwinian concept of evolution, and of the shifting of a period of preoccupation, by the geneticist, with morphological character inheritance to a contemplation of the genetics of plants in nature. Thus, it is the beginning of population genetics. These problems relate themselves to the inheritance of physiological capacity and they treat such capacity as it rests upon environmental factor and physiological function relationships. These factor-function relationships depend upon genetic characters operating through the species population to produce geographic elaboration of variously preadapted individuals throughout the environment. In summation, popu-

lation genetics thus bears upon the major geographic problems encountered in floristics. Obviously, on an experimental level such problems are restricted to cases wherein there is potential gene exchange. Nevertheless, they are important in that they point the way toward the solution of the larger problems of plant geography, every step of which is concerned with the inherent capacity of an individual plant to function within a given environment. These factor-function relationships serve to focus the attention of the plant geographer on the necessity of keeping every aspect of his interpretation on the level of functioning plants in suitable environments, and each of them must be thought of in terms of some possible continuity of individuals and environments. If bridging of environments is contemplated, these bridges, in order to be acceptable, must be compatible with principles of physiology. On the other hand, with recognition of the evolutionary possibilities through genetics and with the realization that not all individuals of the species population are exactly alike, we are provided with much freedom in interpretation. Hence, there is a reservoir of variability in the species population which, through recombination and cytological change, will enable its successive individuals to invade new habitats and occupy new areas.

In his paper, "The genotypical response of the plant species to its habitat," Turesson (44) recorded his researches which lead to far-reaching conclusions concerning the environmental relations of plants. In a series of transplant studies involving what had been presumed to be modifiable ecological variations, he was able to show clearly that these variants were not ecologically modifiable except through genetic processes and that such variations were hereditary to a far greater degree than was ordinarily supposed. This was a truly significant blow to the Lamarkian thinking that had dominated a generation of workers in this field. It clearly meant that a new individual's adaptive ability to meet another condition resided in a preadaptation which may not have been in any way affected by the environment in which it had to function. The role of the environment in moulding the range of variation of populations of individuals is in selecting individuals having cytological and gene combinations favorable to it and rejecting individuals not suited to it. As a matter of degree, one can state that within the variant population favorable gene combinations will be allowed expression, whereas individuals with unfavorable gene combinations will survive only when these are masked in recessive combinations with the favorable. Gene exchange and recombination in the population operates on a statistical basis. We have here a strong force determining the character of variables which can survive in the population and, hence, determining also the character of the genotype. This Turesson speaks of as a "genotypical response" of the species to its environment.

In considering the meaning of his results, Turesson developed the idea of the ecotype as a unit geographic race in selective adjustment with the total significant environment. He regarded it as a group of biotypes within a species (ecospecies) which have arisen as a result of "genotypic response"

to the habitat. Thus, it is a population or a series of like populations in selective adjustment with their special environment.

An outstanding aspect of this concept is that it represented a part of an inclusive unitary system of classification similar to that employed in taxonomy, but presumed to rest, at least in part, on the physiological and genetic behavior of the members of the population. The ecotype was included in the ecospecies, the latter regarded as a unitary expression of the sum of such ecotypes as were able to exchange genes without detriment to the offspring. The ecospecies, in turn, were included in a more nebulous unitary category which he called the coenospecies, and which he presumed included a group of plants "of common evolutionary origin, so far as morphological, cytological, and experimental facts indicate." Although limited gene exchange is construed as possible between members of the same coenospecies, some sterility barriers have developed. Thus, the coenospecies may correspond to a group of taxonomic species or in some cases an entire genus.

The immediate effect of this classification was a preoccupation with the attempt either to fit it into taxonomic systems or to fit taxonomic systems into these biosystematic categories on the assumption that the facts developed by such an experimental attack upon a problem would provide the basis for a "definition" of the species involved. The thought was that in these biosystematic categories there was foundation for both physiology and genetics within the species concept. In fact much of the literature that followed in this field is concerned with what has been termed "the species problem," a problem whose major premise is probably to be found in philosophy rather than in any of the aspects of manipulatory science. Much of this work shows a lack of understanding of the abstract nature of classification and what results from it. However, this method of approach was by no means sterile of results for it led directly to the physiological researches that we find significant to our problem.

A series of papers entitled "Experimental studies on the nature of species" by Clausen, Keck & Hiesey are outstanding. In the first of these (8) this group of workers discuss the effect of varied environments on Western North American plants. Their method of study involved transplanting clonal representatives from distinctively different climatic environments into each of three climatic environments determined by altitude. This was followed by a cytogenetic analysis of the results. They concluded from their large number of experiments that regional differentiation to climate is general, the transplant experiments showing such differentiations to be hereditary and not the result of environmental modifications. Through their cytogenetic analysis they were able to show that each climatic representative reproduced its kind through breeding, and differences existing before or after transplanting appeared to have resulted from both genic and chromosomal differentiation. Work upon *Potentilla glandulosa* showed that both morphological and physiological characters depended upon a series of genes,

and that in some cases morphological and physiological characters were linked. Hybrids between ecotypes of *Zauschneria* showed similar cytological relationships with respect to morphological and physiological characters. In any event, here is a clear case which indicates that climatic races owe their precise capacity for survival in a given climatic complex to genetically controlled characters which, in turn, control physiological processes. Further results are graphically summarized in Figure 1.

The second paper in this series we shall discuss under our treatment of polyploidy, and will consider here the third paper entitled "III. Environmental responses of climatic races of *Achillea*" by Clausen, Keck & Hiesey (10). In this research project the authors pursued more intensively the more promising materials investigated in their first paper. *Achillea* was chosen for their intensive study because of its very wide geographic and ecological distribution. They point out that the *Achillea millefolium* complex, "almost blankets the temperate and subarctic regions of the Northern Hemisphere." Being rhizomatous perennials, the plants lent themselves admirably to clonal division, a point of special value to this kind of research. Eighty-three different clonal units of *Achillea* were collected from areas ranging from the California coast to the timberline in the Sierra Nevada, northward through Oregon, Washington, and Idaho with outlying clones from Seward and Kiska in Alaska, and from Swedish Lapland and Denmark. Physiological differences between the races were evidenced by the fact that some were winter-dormant, a few were summer-dormant, while others displayed no dormancy.

Their experiments were conducted at three field stations (sea level, intermediate, and timberline) having average growing seasons of 283 days, 145 days, and 67 days, respectively, and wherein local native races had become adapted.

An additional experiment was set up at Pasadena in the controlled climate greenhouses of the Phytotron [Went (49)]. The results are depicted in Figure 2. Reactions of the clones to the experimental environments were recorded in terms of length of stems, number of stems, and dates of flowering.

The authors conclude from these experiments that an ecological race approaches equilibrium with its environment through the balance between the natural variation in the population and the forces of natural selection operating in the environment. The race remains flexible through the genetic diversity of its members, which provides it with the capacity to adjust itself to changing environmental conditions. Thus, continuity of the species and its widespread occurrence has come about.

Widespread species must be comprised of many races which arise through natural selection by diverse environmental factors. This selection results in local and regional races that are genetically different from one another and different within themselves. With respect to climatic races they point out that there has been a synchronization with the diurnal and seasonal periodicities of the environment. Furthermore, it is pointed out that fitness
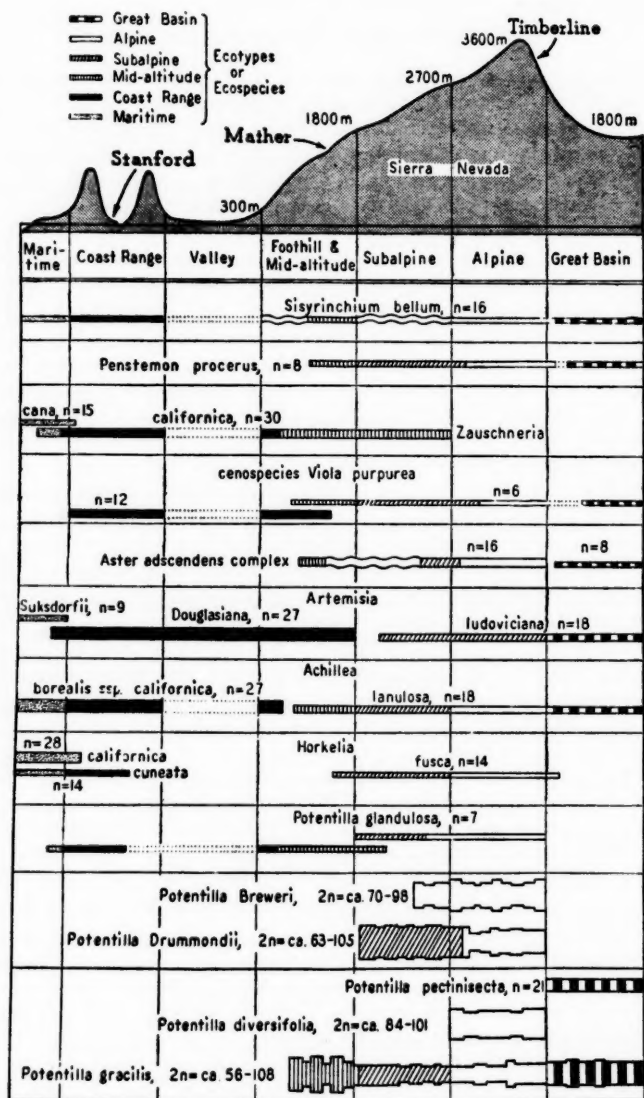
FIG. 1. Distribution of ecotypes and ecospecies in a transect across central California. Dotted lines indicate absence of forms; wavy lines, exact boundaries of ecotypes not established. Width of symbols indicates chromosome number and is roughly proportional to the degree of polyploidy. Names refer to ecospecies. The alpine ecotypes occur only up to about 3100 to 3300 m. altitude. (From Clausen, J., Keck, D. D., and Hiesey, W. M., Carnegie Inst. Washington Publ. 520, p. 419. 1940, by permission.)

FIG. 2. Summary of the physiological experiments in the air-conditioned greenhouses in Pasadena, showing the various experimental conditions, the individuals of *Achillea* used, and the degree of vigor and flowering of the clones. (From Clausen, J., Keck, D. D., and Hiesey, W. M., Carnegie Inst. Washington Publ. 581, p. 89, 1948, by permission.)

is always expressible in terms of the reactions of the individual through its individual tolerances.

From the point of view of physiology and plant geography, these researches by Clausen, Keck, and Hiesey clearly point to the nature of the elaboration of populations throughout environments and their extensions to new environments. The interbreeding population with its varied individuals serves as the reservoir of genetic variables, and the environment, through natural selection, selects variables favorable to it. Through any of several isolating mechanisms of an environment, independent development within their own genetic influence may produce races within the species. These may develop further, producing differences that may best be regarded as species. Thus, it becomes clear that geographic elaboration and speciation result from the same causal dynamics. Beyond this, the geographic problems of genera and families appear to be a function of time duration and the enlargement of gaps between related progeny through these same ecogenetic processes operating in individuals of isolated populations and through local exterminations. The investigations of such problems are precisely the investigations of the inheritance of physiological capacity of individuals in interbreeding populations as these are permitted expression by environmental selection.

POLYPLOIDY

With the discovery of polyploidy as a cytological mechanism in speciation, considerable attention was focused upon its precise role and whether or not it was significant in natural selection. As is usual with new discoveries, more was claimed than could actually be demonstrated when the facts became more fully known. Even so, these overstatements of the case had a definitely useful role in the development of the science, for they at length served to fix the limitations of the role of the phenomenon.

The literature concerning polyploidy has been reviewed by Müntzing (30) and by Stebbins (36, 37, 38) and will not be considered here beyond summarizing the relationship of polyploidy to our problem. Hagerup (14, 15, 16), after considering the geographic and ecological occurrence of polyploids, concluded that polyploids were associated with rigorous environments. Later (17) he modified this view to state simply that a polyploid may occur in a different environment from the diploid. Shimotomai (35), Tischler (40, 41, 42), and Rohweder (32, 33) reported that maritime plants were chiefly polyploids. On the other hand Clausen, Keck & Hiesey (8) found no such relationships in the maritime plants with which they worked. It would appear that the problems resolve themselves into particular ones of special cases. There are valid instances of correlation of chromosome number involving polyploidy and geographic and ecological distribution [Anderson (2); Anderson & Sax (3); Clausen, Keck & Hiesey (8, 9)], but at the same time no generalization can be made as to the kind of environment to which either

polyploidy or diploidy is usually associated. The importance of polyploidy to our problem is that it constitutes one of the genetic mechanisms whereby physiological differences arise, enabling the resulting population to become channeled into a different environment and new area.

Perhaps the best documented research on the role of polyploidy in speciation and attendant elaboration over environments is that of Clausen, Keck & Hiesey (9) in their series on "Experimental studies on the nature of species: II. Plant evolution through amphiploidy and autoploidy, with examples from the Madiinae." The preface is recommended as an annotated glossary of the complicated terminology employed in this field of genetic research.

The Madiinae of the Compositae constitute an enormously complex group of plants whose taxonomic problems by the comparative methods of gross morphology alone seemed beyond resolution. The research of the authors consisted of a cytological analysis of the related species and, where polyploidy was indicated, an attempt to synthesize the polyploid through crossing and thus determine the kind of polyploidy involved. In some cases, an amphiploid was synthesized that is not known to exist in nature, but which had all of the attributes presumed by the authors to warrant its classification as a species. Presumably the geographic isolation of the parents in nature contributed to the failure to produce the polyploid naturally.

With respect to amphiploidy as a method of speciation they conclude that the greatest success as a self-perpetuating population is achieved when the parents are of such a nature that their respective sets of chromosomes will not pair. Hence, in the further meiosis of the progeny the chromosome pairing will be orderly, but to the degree that the chromosome pairing of the progeny deviates from this pattern, the less likely is the progeny to succeed genetically or through environmental selection as well as in "competition" with its diploid ancestors.

The remainder of the paper is largely observational and deals with the occurrence of many natural amphiploids and autoploids, widely selected taxonomically, giving their environmental occurrences and probable origins as reported by various authors. Neither these nor the artificial polyploids treated will be further discussed here, as they are either not fully documented as to results or their natural environmental relationships are obscure and hence are not pertinent.

Another line of research used as a means of explaining distribution deals more directly with the relation of plant growth to the substances which are absorbed from soils. These researches have been chiefly concerned with special distinctive plant populations found in edaphic environments such as serpentine and highly acid soils. Since most of the researches prior to these have emphasized climatic factors as most significant for interpretations of plant geography, the following work may be considered a new departure into the importance of the nutritional factor for plant distribution.

Although researches on **serpentine** soils had emphasized hitherto the

problem of their infertility for crop plants, very little had been done in the way of discovering why plants native to them were able to survive. The researches of Walker and Kruckeberg are outstanding in this field of investigation.

Walker (48) interested himself in the phenomenon of certain native species of plants able to tolerate serpentine soils which were not only completely inadequate for ordinary crop plants grown in the region but which were also restrictive to the native floras of other nearby soils. Areas of serpentine origin are often sharply delimited within distances of a few yards. Walker collected a number of such serpentine soils, and selected several of them for comparative growth studies in the greenhouse using Marglobe tomato, Romaine lettuce, and certain species of plants endemic to serpentine. He observed failure of the crop plants to develop without acute manifestations of nutritional abnormalities. However, his native species of *Streptanthus* appeared to grow normally. From chemical studies of the soils it was revealed that an unusually high amount of magnesium, compared to calcium, existed in their soil solutions and in their cation exchange complexes. It was suggested from the stunted growth of the tomatoes and lettuce that they were deficient in calcium. Chemical analysis of the plants also showed low amounts of calcium and unusually high amounts of magnesium. Chemical reconstitution of his soils, i.e., replacing magnesium in part with calcium verified this suspicion. Comparative studies of the native species of *Streptanthus* disclosed that magnesium had not been absorbed by these native species in quantities even relatively comparable to those accumulated by tomato or lettuce.

An interesting by-product of Walker's studies was the disclosure that the reaction pattern of the cultivated plants was very uniform as to growth and symptomatology of the nutritional disease, whereas the rate of growth of the wild species was highly variable among individuals, demonstrating the genetic variability of the wild species as contrasted with the crop plants. In other words, the cultivated plants were as near to pure lines as we usually get in crop plants, while the wild species were probably highly heterozygous.

A third important discovery made by Walker had to do with soil molybdenum levels. He observed that, after fertilization of the serpentine soils with nitrogen, potassium, phosphorus, and calcium, severe molybdenum deficiency was shown by the crop plants but not by *Streptanthus*. Indeed Walker's report (47) was the first one to show the presence of a molybdenum-deficient soil in the Western Hemisphere.

The plant geographer finds in Walker's researches at least a partial explanation of endemism on serpentine soils. It rests in the capacity of the native plants investigated to absorb selectively an adequate amount of calcium from high magnesium soils, and at the same time to avoid accumulating magnesium in the high quantities absorbed by the crop plants. Plants with such capacity for differential selection of calcium and which are also able to tolerate other local conditions characteristic of the shallow serpentine

soils will be selected by these environments. There is, however, no suggestion in Walker's results as to why these species are confined to serpentine. His work explains only why these species can grow there while others will not.

Kruckeberg (19) concerned himself with extending this problem by testing the adaptive character of several species which occurred both on serpentine soil and on other soils. Among these were *Streptanthus glandulosus* Hook., *Gilia capitata* Dougl. and *Achillea borealis* Bong. In each case it was demonstrated that the plant-soil relationship was determined by the genetic attributes of the plant. Kruckeberg showed that the nonserpentine races showed marked intolerance for serpentine soil. There were a few exceptions noted among individuals of *Achillea borealis* from nonserpentine soils. These appeared able to grow on both kinds of soil. Kruckeberg concluded from these studies that there is a fundamental aspect of the variation of plants that is physiological in its expression but which becomes apparent in the distribution of the species populations over varying soil types.

With respect to *Achillea borealis*, Kruckeberg was dealing with a species of demonstrated ecotypic character [Clausen, Keck & Hiesey (10)] in response to segments of the climatic gradient across California, and in turn he was able to demonstrate that this climatic ecotype was further divisible into edaphic ecotypes of various kinds. The implication of this on the nature of the ecotype is far-reaching for, as Kruckeberg pointed out, we are dealing with ecotypic variation within and across natural populations rather than with mutually exclusive ecotypic units. With respect to some of these variations we can establish significant taxonomic units. With other race variants the same individuals will be a part of different ecotypic patterns of a noncoincident geographic character and hence may not fall into line with a desired taxonomic segregation.

In another aspect of this study, Kruckeberg (20) devised a mass selection experiment. He mixed seed of many serpentine endemics and many nonserpentine plants and broadcast them over prepared beds of both serpentine and nonserpentine soil. His results were an actual demonstration that out of the mass of seed presented to a soil environment the soil may select its biota. The results disclosed that whereas all of the seeds germinated on the nonserpentine soil, the serpentine species did not develop far beyond germination. On the serpentine soil only the serpentine species germinated. The dying of the serpentine plants on the nonserpentine soil was interpreted as being the result of "competition," although what aspect of "competition" was involved was not determined. Certainly, since the nonserpentine species did not germinate on serpentine soil no aspect of competition could be involved in this case.

Through his clarification of the ecotype concept Kruckeberg's work also provides clear evidence that the physiological characters of plants are heritable on a unit character basis as are morphological characters. The relationship of one function to a given environmental condition is inherited inde-

pendently and probably through different gene combinations than are other factor-function relationships. Thus, it becomes apparent that what at one time was generally spoken of as "broad tolerances" of a species must now be looked upon in terms of a variable species population. Even within a single local interbreeding population the span of the conditions occupied is probably greater than the capacity of any single individual to survive everywhere in the area of the population. Local and minor variation is prevalent even on this level and environmental selection is operating. A population is an ecotype only to certain environmental conditions, and portions of the same population may be ecotypic to other conditions of a different geographic pattern. In both cases, the relationship is genetic through a preadaptation and its manifestation is through environmental selection.

Melchers (29) concerned himself with a study of two species of *Hutchinsia*, differing largely in petal shape but occurring on different soil types. *Hutchinsia alpina* is, so far as is known, confined to soils derived from limestone while *H. brevicaulis* occurs only on igneous rocks. In artificial cultures it was demonstrated that the latter species requires less calcium than does the former. A genetic analysis clearly showed that the inheritance of these physiological characters was associated with a relatively small number of genes. The occurrence of individuals having a corolla type of *H. brevicaulis* but occurring on limestone would indicate that although the morphological and the physiological characters usually occurred together their inheritance was independent of each other.

Venogradov (46) experimented with the environmental requirements of seedling establishment in races of *Pinus sylvestris* L. This species occurs both on sandy river terraces and on calcareous soils. It was demonstrated that although both races germinated equally well on both soil types, further development of the seedlings was inhibited by conditions present in soils other than the type on which the parents were growing.

Billings (4) investigated the island-like occurrences of forests, which represented an eastward extension of the flora of the Sierra Nevada, into what was presumed to be a climatic environment which would support only sagebrush. His preliminary work disclosed that these were, in effect, edaphic islands characterized by hydro-thermally altered igneous rock yielding soils of acid character and deficient in phosphorus and nitrogen as well as in the exchangeable bases. Although his research was designed to discover the nature of the deficiencies and not to explain how the present special vegetation overcame these deficiencies, it does serve the objectives of plant geography by explaining why the normal vegetation of the area does not invade these specialized situations. This is accomplished by the use of native wild plants for the experimental study in conjunction with certain cultivated plants. For this purpose the sagebrush, *Artemisia tridentata* was used.

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PHOTOSYNTHESIS[1,2,3]

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INTRODUCTION

The unsurpassed pace of photosynthesis research in the last year has made our recent review entirely inadequate (1). This paper is based on the older review and, like it, must be severely restricted in coverage. Emphasis will be placed on the newer findings on the photochemistry of photosynthetic pigments, chloroplast structure and composition, energy transfer mechanisms, the Hill reaction and its relation to photosynthesis. A short section on quantum requirements is necessitated by the abundance of work along these lines. The review concludes with a very brief discussion of the most recent papers in areas of work not included in the above topics. We will occasionally take the liberty of commenting on the possible significance and reliability of the results reviewed. For more detailed information and coverage, and for other points of view, the reader is referred to the excellent older reviews of Rabinowitch (2), Hill (3, 4), Whittingham (5), Franck (6), and French & Milner (7) together with the recent reviews of Brown & Frenkel (8), Moyse (9), Pirson (10) and Ueda (11).

PHOTOCHEMISTRY OF THE PIGMENTS

Structure and theory.—Though no precise quantitative predictions about the chemical properties of large, conjugated molecules can be expected from the application of quantum-mechanical theory for some years to come, theoretical calculations offering considerable qualitative promise are now an active research undertaking. Chlorophyll, porphyrin, and carotene molecules have been considered in regard to their electronic excitation states, charge distribution, and polarizabilities together with the effect on these character-

istics of substituent groups [Platt (12); Takeda & Oki (13); Araki & Murai (14)]. Chemists are also becoming increasingly interested in the weak and generally nonspecific intermolecular interactions so important in living organisms. While these are usually attributed to "van der Waals forces," a new and probably very important type of interaction due to transient electron transfer between partners has been suggested by Mulliken (15).

The appearance of improved spectrometers has initiated a reinvestigation of the structure of pigments by infrared methods as described in a paper by Weigl & Livingston (16). Among other conclusions, they confirmed the proposal of Fischer & Strell (17) that allomerization of chlorophyll-a introduces an alkoxy group at position 10. Kato et al. (18) have presented the newest interpretation of the electronic spectra of chlorophyll [see also Stupp & Basel (19)]. While all such observations are not of immediate utility to the plant physiologist, they provide necessary clues for the theoretical analysis of the electronic structure of excited and unexcited pigment molecules and thus to their chemical reactivity.

In vitro reactions of chlorophyll.—As will be discussed in a succeeding section of this review, the electronic quantum of energy introduced into the pigment system of photosynthesizing organisms on absorption of a photon wanders about among the pigments until it reaches some particular chlorophyll-a molecule (or bacteriochlorophyll molecule in bacteria). From these molecules the quantum is converted by some unknown process either into electronic energy of some nonpigment participant, or into the energy of chemical bonds. It is thus unnecessary to give much attention to the photochemical reactions of pigments other than chlorophyll-a and bacteriochlorophyll, although we should not ignore the possible chemical involvement of the carotenes.

Livingston & Weil (20) now concur with Evstigneev et al. (21, 22) in the belief that activators of chlorophyll fluorescence exert their effect through the magnesium atom. There can be little doubt of the correctness of this interpretation since only metal tetrapyrroles show activation and, of these, the magnesium chlorophylls alone show large effects, even when allomerized. Magnesium phthalocyanine, which lacks the pentanone ring, behaves like chlorophyll, whereas, the pheophytins show no effect [but see (22)]. Thus, the magnesium atom with its associated activator has the same fluorescence effects as the two hydrogen atoms of the pheophytin since the latter fluoresces well in both polar and nonpolar solvents. Freed & Sancier (23) have observed that the pheophytins also form addition products with solvents and other activators.

Generalized bases of all types in nonpolar solvents or even slightly polar solvents free of additions raise the fluorescence yield of chlorophyll-a to the same maximum value of approximately 25 per cent [Forster & Livingston (24)]. Water is very effective, so that very dry nonpolar solvents must be used to prevent activation, under which conditions the fluorescence is essentially zero; indicating loss of electronic energy to heat in less than 10^{-12}

sec. (23, 25). This means that nonactivated chlorophyll possesses one or more electronic states connected each to the other and to the "singlet" state to which it is excited by red light. One of these states, in turn, is connected to the original ground or unexcited state. By this chain of states the excess electronic energy held by one or two electrons moving in orbitals or pathways in space farther removed from the nuclei than in the ground state is converted to motion of the nuclei, i.e. vibrational energy. This energy is quickly lost to solvent molecules by collison. By connected states we mean simply that the two electronic states have at least one common identical configuration of the nuclei which they reach in the vibrations of these nuclei moving in the electrical field of the electrons. Thus, when the molecule with its electrons in one state achieves a nuclear configuration identical with one possible for another electronic state, there is some probability, governed by known factors, that the electrons will jump to new orbitals to put the molecule as a whole into the second state. This process is known as crossing.

The conversion of electronic energy to vibrational energy is known as internal conversion. It can occur when a chain of connected states starting from the originally excited state contains the ground state, thus allowing re-access to the ground state without emission of energy in photons. The crossing point allowing reaccess to the ground state is usually a very distorted or uncommon one for the normal electronic ground state which is not usually excited to very great vibrational motion. The configuration corresponds to a separation of the nuclei which is characteristically reached only by an extreme vibration. Thus, after crossing, the molecule finds itself in a high-energy vibrational state where the energy is stored in extreme stretching and compressing of the molecular springs. But in solution this situation is unstable, since the vibrational energy of a molecule thus excited is greater than that of solvent molecules and the excess leaks away to solvent molecules after a few collisions, each of which occurs in about 10^{-13} sec.

Activated chlorophyll molecules do not have a rapid process of internal conversion. This is demonstrated by the fact that they persist in the singlet excited state to which they were originally excited for the relatively long time required for fluorescence to occur. For chlorophyll-a this time is about 2.5 $\times 10^{-8}$ sec., on the average. This may be calculated from the lifetime of this state given as 7×10^{-9} sec. by Livingston & Weil (20) and a fluorescence yield of 25 per cent measured by Forster (26) and by Livingston (24). The lifetime in the excited state is thus lengthened at least 7,000 times by fluorescence activators. All activators, such as the alcohols, amines, nitrogenous bases, etc., act in the same manner, although their association equilibria may vary. The highest value measured by Livingston & Weil was $1.6 \times 10^{-5} M^{-1}$ demonstrated by heptylamine.

The same authors noted that in the presence of activators the spectra shifted slightly and the light absorption coefficients increased by about a factor of two at the major peaks. Evstigneev et al. (22) noted that different activators produced slightly different spectral shifts in pheophytin as well

as chlorophyll. Freed & Sancier (27, 28), working from an entirely different point of view, noted similar spectral changes on cooling chlorophyll and related pigments in nonpolar solvents to rather low temperatures. Recently, they have been able to show that these changes are due to the association of activator. Since the activators used (which were either trace impurities or, strangely enough, very weakly polar molecules) were poor, and since the heat of association of all activators is positive, the association compounds were found only at low temperatures. These studies [Freed & Sancier (23)] showed that the different peaks of the spectrum increase in different ways. In addition, they were able to study the association of a second molecule of the activator even though this association was very weak. Miller & Dorough (29) had previously observed that magnesium tetraphenylchlorine forms both mono- and dipyridinates even at room temperature when this good activator is strongly associated. Apparently an activator molecule can attach to both sides of the magnesium atom to complete a coordination number of four. However, both Freed and Sancier and the Evstigneev group noted that the pheophytins also form the complexes. Since it is the basic nature of the activator which governs its association, we must conclude that both the magnesium atom and the hydrogen atoms which substitute for it in the pheophytin are largely held in ionic linkages which preserve their strong electropositive character. That is, they are primarily in the cationic condition. Probably magnesium is a more effective donator of electrons to the organic portion of the molecule than the hydrogens, but the effect on the absorption spectrum when magnesium replaces the hydrogen atoms is not large (25).

Only with activators do we observe a large effect in the interaction of chlorophyll with light. Even then only the fluorescence is markedly affected, since the absorption spectra shifts but slightly and the amount of light absorption changes by a relatively small factor. The position of the spectral peaks is determined by the relative energy separation of the ground and first excited state. We need not consider the excited singlet state achieved under illumination with blue light since the extra energy, which is probably vibrational, is rapidly lost to drop the molecule into the red singlet state we have been considering. We must conclude then that activators attached to chlorophyll magnesium atoms replace the crossing point available in the nonactivated molecules by a less efficient crossing point which forces the photoexcited molecule to remain in the singlet state for a much longer time.

Livingston & Ryan (30), employing an elegant flash-scanning technique, observed that fully-activated chlorophyll-a on high-intensity flash illumination undergoes transient changes in its absorption spectrum consisting of a partial bleaching. Under lower-intensity continuous illumination another series of changes were observed. While these changes have been anticipated in older work, the recent study provides quantitative data sufficient to work out the kinetics of the process. The authors interpret the results to indicate (a) a metastable excited state decaying in a bimolecular reaction with unexcited chlorophyll molecules with a rate constant of $4.5 \times 10^9 M^{-1}$, and (b) a

formation of radicals or ions by a reaction of the metastable state with solvent. The rate constant given above is that for chlorophyll-*b*, but chlorophyll-*a* behaves in a similar fashion. The lifetimes of these states are much greater than that of the first singlet excited state, particularly at low concentration or in rigid media where, since collisions are limited, both states will last for some time.

Of considerable interest is the observation, consistent with many other observations on the effect of oxygen on chlorophyll, that neither state appears in the presence of air. Thus, oxygen must prevent the formation of the metastable state (*a*) by tying up the singlet state; (*b*) by providing a rapid pathway for reversion of the metastable state to the ground state; or (*c*) by producing a long-lived chlorophyll-oxygen compound with spectral properties nearly identical with those of ground-state chlorophyll (no spectral changes of any sort were observed in the presence of oxygen). Livingston & Ryan (30) favor the second alternative, i.e., a rapidly decomposing compound which oxygen makes with chlorophyll in the metastable state. The first alternative is unlikely since oxygen does not influence the absorption spectrum and thus does not change either the ground state or the singlet excited state. This is not surprising since the excited singlet is probably a state of separated charge, i.e., negative charge at one point, positive charge at another, and thus is not likely to react with the normal oxygen molecule which possesses two unpaired electrons. Oxygen is known to be a very efficient fluorescence quencher (25). This is equivalent to saying that oxygen reduces the time spent in the singlet excited state by improving the process of crossing out of this state. It may do so either by combining with the subsequent state, in which case the crossing point is lowered, or by assisting in the uncoupling of electrons necessary in the electronic rearrangement at the crossing point if the subsequent state has a different number of unpaired electrons than the singlet excited state (which has none). The latter function will increase the probability of crossing. Since the oxygen molecule normally possesses two unpaired electrons which makes it a triplet or diradical, and since there is much evidence to suggest that the metastable state of chlorophyll is also a triplet, oxygen probably functions both ways to induce crossing. Of particular interest is the possibility that the high energy oxygen-chlorophyll compound thus formed may possess sufficient life expectancy to serve as the mediator for the photosensitized reactions of chlorophyll and other pigments *in vitro* and perhaps *in vivo*. We shall return to this matter after considering some recent studies of the *in vitro* reactions of chlorophyll.

The spectrum of the "radical" state observed by Livingston & Ryan (30) is similar to that of the Molisch brown phase of chlorophyll. Freed & Sancier (23, 31) also observed this "phase test" spectrum in their solvation studies when isopropylamine was the activator. Presumably, the latter substance associated not only at the magnesium atom but at carbon 10 as well. Watson (32) obtained a similar spectrum by treating alcoholic chlorophyll solutions with ferric ions and other oxidizing ions. In contrast to many

older studies in which the same observation was made, he secured complete reversibility by adding reducing substances like cuprous chloride within a short time. Without this addition, or after some delay, the characteristic green spectrum of allomerized chlorophyll appeared. Watson favors the explanation that ferric ion bleaches by oxidizing chlorophyll, as was generally concluded from the older experiments. The ultimate site of oxidation occurs in ring V. Water accelerates the irreversible allomerization.

The spectrum of the Livingston & Ryan "radical" (30) is also similar to that observed by Linschitz & Rennert (33, 34), which results when chlorophyll is illuminated in the presence of certain quinoid substances in a glassy medium at very low temperatures. Under those conditions the quinones, or even solvent molecules, receive one electron from excited chlorophyll. Because of the rigid state of the system, the oxidized chlorophyll can be preserved indefinitely. Similar results were obtained by Kachan & Dain (35) and by Krasnovskii (36) who worked under the more difficult conditions occasioned by higher temperatures. Pheophytins participate poorly in reactions of this type and allomerized chlorophyll does not participate at all. Thus, the presence of both magnesium and an intact ring V in the pigment are required. The iron analogue of chlorophyll appears to carry out similar photoreduction reactions [Ashkinazi & Dain (37)].

Another reaction involving the oxidation of chlorophyll is the chemiluminescence process studied by Linschitz & Abrahamson (38). In the presence of tetralin hydroperoxide and certain other peroxides at high temperatures chlorophyll undergoes a series of reactions thought to be the following where DH_2 is the pigment:

$$\diagdown \!\!\!\! \overset{H}{\underset{}{COOH}} + DH_2 \rightarrow \overset{H}{\underset{}{CO\cdot}} + DH\cdot + H_2O$$

$$\diagdown \!\!\!\! \overset{H}{\underset{}{CO\cdot}} + DH\cdot \rightarrow DH_2{}^* + \diagdown \!\!\!\! C{=}O$$

$$DH_2{}^* \rightarrow DH_2 + h\nu$$
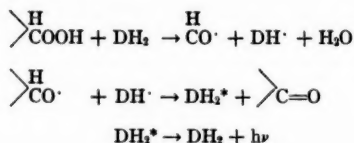
The chlorophyll is slowly destroyed in side reactions. The similarity of this scheme to the other oxidations is obvious if operated backwards, and it provides a possible mechanism for energy conversion in photosynthesis.

Evidence favoring the triplet nature of the metastable state or the occurrence of a subsequent true radical appears in Uri's (39) studies of the initiation of the polymerization of methyl methacrylate which is known to require radicals. Chlorophyll in the activated state initiates polymerization upon illumination. Oxygen destroys this ability as does ferricyanide ion, but ascorbic acid and thiourea greatly increase the amount of polymerization. The latter two compounds are particularly good substances for reducing illuminated chlorophyll, but there seems no necessary reason to suppose that all of the radicals detected by Uri's technique were formed by the same reaction.

Most, if not all, of the above reactions which occur in the absence of oxygen can probably be related to the "radical" state of Livingston & Ryan (30), as can the slowly reversible oxidative bleaching obtained previously with high intensity illumination [reviewed in (40), p. 486 *et seq.*]. Much work will be required to correlate all of the confusing data which have accumulated with studies involving the variation of activator and solvent, the addition of oxidizing or reducing agents, the addition of salts, etc. The wide diversity of results reported undoubtedly has a single, simple basis resting, perhaps, on the relative ionization energy of excited chlorophyll and activators or other additives. For present purposes it is sufficient to note that all the reactions in which chlorophyll is oxidized are poor ones in the sense that the quantum yields are low and the products unstable. We shall see that the same may be said for reductions of chlorophyll.

Reductive bleaching is a characteristic reaction of many dye molecules. However, until recently, this process has not been so well established for chlorophyll as has the oxidative bleaching reaction. In basic solvents like pyridine, photo-excited chlorophyll can be reduced by ascorbate, hydrogen sulfide, phenylhydrazine, dioxymaleate, and cysteine in a process which has been extensively studied by the Krasnovskii group (41 to 44). These reactions are discussed in more detail in Rabinowitch (2). Since the latter compounds are fairly strong reducing agents, the excited chlorophyll is obviously not readily reduced and, in fact, the reduced chlorophyll is in turn reported to be able to reduce diphosphopyridine nucleotide (DNP), riboflavin, and safranine. These latter reactions, however, can only be achieved by employing, in addition to the reducing free energy of the reductant, some of the free energy originally stored in the chlorophyll on photoexcitation. In strongly basic organic solvents (pyridine, etc.) reduced chlorophyll reverts readily to its original state. Strangely enough, the spectrum of the reduced molecule is similar to that of Livingston & Ryan's "radical" (30). The ability of various chlorophyll preparations to undergo photoreduction parallels their relative fluorescence intensity even when they consist of detergent-dispersed chloroplast fragments (45). Reducing agents generally fail to quench chlorophyll fluorescence, but oxidizing agents including oxygen and quinone are quenchers in activating solvents [Livingston *et al.* (11 to 27)].

Bacteriochlorophyll and bacteriopheophytin are also reduced under the conditions described above [Krasnovskii & Voinovskava (44, 47)], but since the latter substance lacks a metal atom, its reduction is either a different process from the others, or the unlikely conclusion must be drawn that magnesium is not involved in any of the cases. These substances can also be photooxidized in the presence of oxygen to give peroxide-like intermediates. Another strange behavior of bacteriopheophytin has been reported by Weigl, who found that the spectrum of this compound is not sensitive to pH changes in contrast to the behavior of pheophytin-*a* (48). The latter substance shows distinct interchangeable acid and neutral forms, but it undergoes an irreversible reaction in basic solutions to a form whose spectrum

differs but slightly from that of the neutral form [Livingston and co-workers (49)].

There appears to be no evidence to support a connection between fluorescence activators and reducing substances. Evstigneev & Gavrilova (50), while studying the influence of various substances on the photooxidation of chlorophyll in toluene, noted that water, ethanol, aniline, and quinoline accelerated oxidation. This indicates the need for activation, as would be presumed. Ether and acetone, which probably did not activate in the concentrations present, had no effect. Pyridine and some other nitrogenous bases retarded the reaction. On the other hand, the latter group of substances greatly improved the photoreduction reaction (51). These observations are intriguing but not yet easily understood. Weigl & Livingston have established (a) that no hydrogen exchange takes place between chlorophyll and water in organic solvents (52), and (b) that no permanent hydrogen exchange takes place between chlorophyll and deuterated ascorbic acid when the latter reduces butter yellow in a chlorophyll-photosensitized reaction (53). Becker & Sheline (54) have been unable to detect an exchange of magnesium between labeled magnesium chloride and chlorophyll. This is in partial agreement with the older work.

Like the photooxidation of chlorophyll, the photoreductions are inefficient processes, perhaps even more inefficient since, chlorophyll appears to be more readily oxidized than reduced. All of the reactions produce unstable intermediates with altered spectra. On the other hand, many of the chlorophyll-photocatalyzed oxidation-reductions such as those involving ethyl thiourea, thiourea, or phenylhydrazine plus oxygen etc., proceed easily with high quantum yields (40). In these reactions the reductant and oxidant are both present, and chlorophyll demonstrates no long-lived intermediate state. As a possible explanation we may note that the only single type of reaction of excited chlorophyll molecules which appears to proceed rapidly in high yield is the type involving oxygen or related substances (which are probably distinguished by their ability to quench fluorescence). Unless this type of reaction can be assumed to be an intermediate step in the *in vitro* photosensitization reactions, the very high efficiency of the latter is difficult to explain, particularly in reactions involving molecular oxygen, since, according to Livingston & Ryan (30), oxygen eliminates or greatly shortens the lifetime of the metastable state which appears to be required as the reactive reservoir of stored energy. For instance, the photooxidation of allylthiourea which was extensively studied by Gaffron (55), demonstrates a high quantum yield weakly dependent on oxygen concentration even at oxygen concentrations thirty-fold weaker than the allylthiourea concentration. On the other hand, the dependence of quantum yield on allylthiourea concentration is large up to concentrations of about 0.01 M. Here again is indicated a very efficient interaction of oxygen and excited chlorophyll molecules, although mechanisms have been proposed for this and similar reactions of chlorophyll,

as well as other sensitizing pigment molecules, which require no interaction between oxygen and sensitizer whatsoever. It would be out of place to discuss here in any detail the various older mechanisms of photosensitization, all of which are skillfully presented in much detail by Rabinowitch (25), but some introductory comments are necessary before describing a new mechanism recently presented by Schenck (56 to 59).

According to Gaffron, Weber, Warburg and Schöcken and others, the photosensitizer transfers its energy to the reductant which then reacts in its long-lived excited electronic state with oxygen. According to Kautsky and others, the sensitizer transfers its energy to oxygen, which then reacts with the electron-donating molecule. Neither scheme involves any electron transfer from or to the sensitizer. Another mechanism proposed by Weiss, however, does require electron transfer from sensitizer to oxygen with an ultimate return of electrons to sensitizer from reductant. In view of the findings of Livingston & Ryan (30), this last would appear to be a poor process for chlorophyll for which oxygen, when present, supersedes other possible reactants but produces no detectable change in spectrum. It should be remembered that the "radical" state of Livinston and Ryan is probably an oxidized state and yields a distinctly changed spectrum. None of the above mechanisms requires compound formation either with oxygen or reductant. There is little evidence favoring a chlorophyll-reductant compound; reductants may be fluorescence activators but they do not quench, and the quantum yield for their photooxidation continues to increase at concentrations far greater than those required to give full activation. On the other hand, there is much evidence to suggest that a fairly long-lived compound is formed between oxygen and some excited electronic state of chlorophyll or certain other sensitizers. The most striking and most recent evidence comes from the long series of papers by Schenck [(56 to 59), together with others listed (58), which is a general review]. The intriguing and partially proven general reaction scheme presented by Schenck on the basis of many studies of photosensitized reactions consists of the following sequence of steps:

$$S \text{ (sensitizer)} + h\nu \rightarrow S^*$$

$$S^* \rightarrow S_r \text{ (diradical)}$$

$$S_r + O_2 \rightarrow O_2S \text{ (unknown structure)}$$

$$O_2S + A \text{ (acceptor)} \rightarrow AO_2 + S$$

$$S_r \rightarrow S; \text{ (poor)}$$

$$S_r + A \rightarrow S + A; \text{ (good)}$$

$$S_r + A \rightarrow A \text{ (oxid)} + SH; \text{ (poor)}$$

$$S_r + AO_2 \rightarrow \text{oxidative bleaching; (poor)}$$

$$O_2S \rightarrow S + O_2; \text{ (poor)}$$
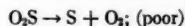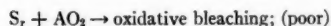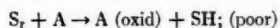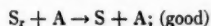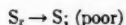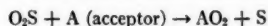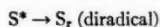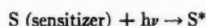
The scheme bears marked similarities to that of Livingston & Ryan (30); neglecting their "radical" state. This mechanism is appropriate for eosin, methylene blue, chlorophyll and certain other sensitizers which can transfer oxygen molecules with good quantum yield (see Illustration A). Only sensitizers of the quinone type which form oxygen-oxygen diradicals R⟨$^{O'}_{O'}$ on excitation preferentially react with electron donors to dehydrogenate them before reacting with oxygen. When oxygen is absent, the "phototropic diradical," S_r, may dimerize and remove hydrogens from other molecules, or lose hydrogens to other molecules. Aerobically the transfer of oxygen to

α - terpinen   askaridol
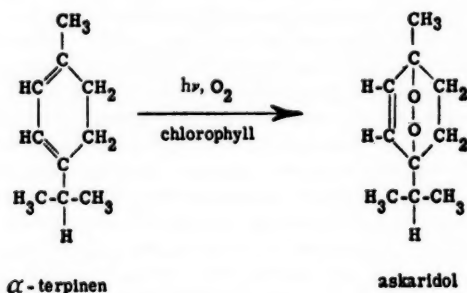
Illustration A

acceptor proceeds very efficiency at sufficiently high acceptor concentrations. The acceptor may retain the oxygen as a bridge (askaridol), form a stable hydroperoxide if no bridge is possible, or undergo oxidative rearrangement. The sensitizer is supposed to add oxygen as a bridge if its diradical state places the unpaired electrons on carbons appropriately spaced for the oxygen molecules. When this is impossible, or when the unpaired electrons lie on carbon and oxygen or carbon and nitrogen atoms, a hydroperoxide diradical appears. Thus for anthracene and eosin the reactions occur as seen in Illustration B. The oxygen-bridged compounds are poor dehydrogenating agents, but they can undergo rearrangement leading to oxidation of the bridged molecule. The peroxidic oxygen compounds are also poor dehydrogenating agents presumably because they go over into the zwitterion form (see Illustration C). The reaction with water is postulated. At low concentrations of acceptor the peroxides react with solvent. Sensitizers with oxygen bridges are quite stable and do not lose the oxygen readily by any unimolecular process.

Kinetic experiments and a very wide range of quantitative studies appear to support Schenck's mechanisms, which allow a clear division of sensitizers

anthracene

eosin
(Na salt)

Illustration B

Illustration C
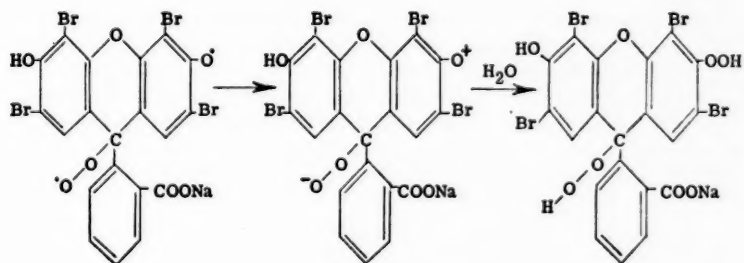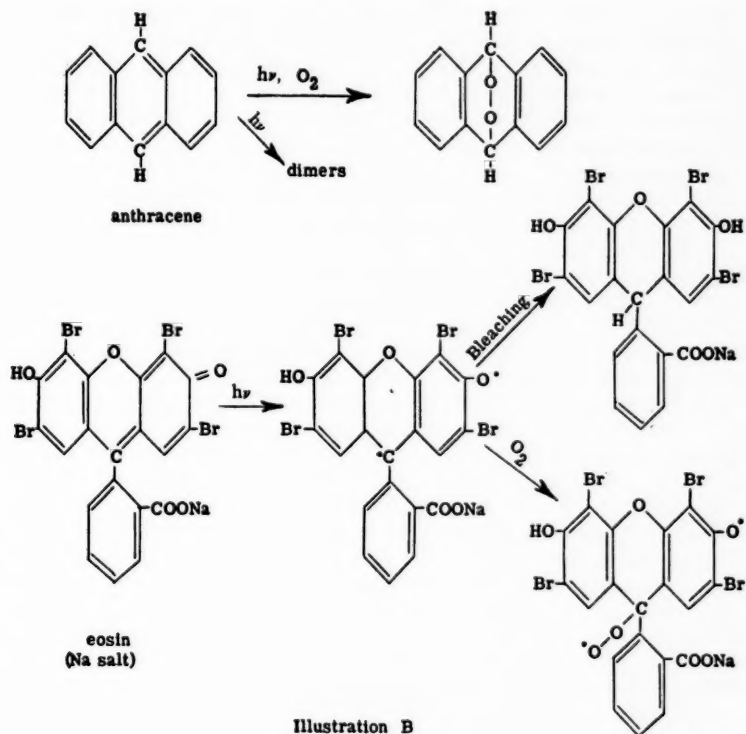
into classes according to the expected position of the unpaired electrons in the diradical states. Considerable uncertainty exists as to the expected life-times of the oxygen compounds of these radicals, particularly for chlorophyll, for which no phosphorescent state has been established and which appears to have no spectrally detectable long-lived state formed following attack by oxygen. Space does not permit a more extensive criticism of Schenck's theories which we believe to be not quite proved by his experiments. Nor should we overemphasize his mechanisms in comparison with the others briefly described [for instance see Förster (60)]. It must be emphasized however that his approach is very promising in that it provides a possible long-lived, high-energy state of chlorophyll consistent with the known reac-tions of oxygen and photosensitizers.

Now what has all this to do with natural photosynthesis? Evidence to be presented, while contradictory, indicates that oxygen is not required for photosynthesis. It would be convenient for the elucidation of the energy relations if oxygen were required, but since this does not appear to be the case, photochemical studies of pigments in the presence of oxygen may have no bearing on photosynthesis. At least the lack of oxygen participation makes it unwise to consider here the mechanism given by Schenck (59) for photo-synthesis since it depends on the cyclic participation of oxygen. On the other hand, there seems to be no reason why oxygen need be considered other than as a prototype molecule. It is a very efficient quencher of fluorescence since it appears to react with the excited pigment molecule on the first collision. How-ever, many other substances are good quenching agents. For instance, Schneck noted that cyclooctatetraene reacts just like oxygen with the diradical sen-sitizer molecule. Certain quinones may well resemble oxygen for this reaction, and both dehydroascorbic acid and 6,8-thioctic acid bear considerable re-semblance to oxygen and are known to be present in chloroplasts. An inter-mediate of the oxygen type, perhaps one of the above, acting as the pros-thetic group of an enzyme could provide a very good vehicle for the conversion of electronic energy into chemical-bond energy and one in which chlorophyll itself would neither gain nor lose electrons. In any event, Schenck's ideas are important for the plant physiologist in that they bear on the photooxidation process *in vivo* and on the large effect of oxygen on fluorescence *in vivo* [Shiau & Franck (61)]. The latter phenomenon demon-strates a dependence of photosynthesis on oxygen even if no actual partici-pation of oxygen occurs in the process. We will continue with this topic in a subsequent section.

CHLOROPLAST STRUCTURE AND COMPOSITION

This section of the review will deal with recent work on the fine structure of chloroplasts, the chemical composition of chloroplasts, the enzymatic activity of chloroplasts and chlorophyll-protein associations. The isolation of pigments, the biosynthesis of chlorophyll and a few miscellaneous related topics will be considered very briefly.

Chloroplast structure.—The investigation of the submicroscopic organization of chloroplasts indicates that they have much in common with other layered lipoprotein biological structures such as retinal rods and nerve myelin [Thomas *et al.* (62); Steinmann (63, 64); and Finean *et al.* (65)]. This type of structure may be intimately involved in the necessary energy transfer procedures as described in the section on energy transfer mechanisms. The work on chloroplast structure has been reviewed by Granick (66) and more recently by Weier & Stocking (67). The present work will serve only to bring these publications up to date. Most of the subsequent papers have been concerned with studies on chloroplast structure by use of the electron microscope. This device has been developed into a powerful tool for investigations of structure on the size level found in chloroplasts. There is fairly general agreement among the different workers concerning the interpretation of electron micrographs of chloroplasts. Differences which do exist appear to result from differences in fixation and other preparative techniques, which are very critical, and differences in age and species of plants used. The results obtained with the electron microscope agree as a rule with the earlier suggestions of Frey-Wyssling and others [summarized in (68)] which were based upon birefringence studies and upon observations with polarized light and ultraviolent light. The model structure proposed on the basis of this earlier work assumed the chloroplast to have a lamellar structure in which thin parallel layers of chlorophyll molecules alternated with thicker layers containing proteins and lipoproteins.

The chloroplasts of those flagellates and algae which have been investigated with the electron microscope show a lamellar structure in which the lamellae extend throughout the chloroplast. The chloroplasts of *Euglena* and of the chrysomonad *Poteriochromonas* have been extensively investigated by Wolken & Palade (69, 70). Each chloroplast is made up o f about 20 dense uniform lamellae averaging 250 Å in thickness which are separated by less dense layers of material ranging from 300 to 500 Å in thickness. These layers are usually homogeneous, although occasional dense spherical granules are observed in them. The apparent spacing of the lamellae was influenced by the preparative steps, in particular, by the fixation process. These flagellates became colorless in about 7 days when grown in the dark. It was found that the chloroplasts of *Euglena* totally disappear in darkness, while those of *Poteriochromonas* shrink and partially or completely lose their laminations. On re-exposure to light chloroplasts with laminations reappear in *Euglena* in approximately 4 hr. The laminations increase in thickness and in number so that by 72 hr. the chloroplasts have regained their normal appearance. Spectrophotometric studies show that chlorophyll is not completely lost in the dark. Upon reillumination some of the remaining chlorophyll is destroyed before chlorophyll synthesis starts. Wolken & Schwertz (71) showed that the data on the chlorophyll content and fine structure of the chloroplasts of the two algal flagellates described above are compatible with a model structure in which the chlorophyll is arranged in monomolecular

layers at the interfaces between aqueous protein layers and lipid layers. The model developed predicts ratios of chlorophyll to other pigments which bracket the majority of experimental values available. Steinmann (63) reported a similar layered structure for the chloroplasts of *Spirogyra* with dense lamellae 70 Å in thickness.

Chloroplasts of the higher plants have been found in general to contain somewhat cylindrical grana which show a lamellar structure [Thomas *et al.* (62); Steinmann (63, 64)]. Most of the available evidence indicates that the chlorophyll is confined to the grana (67). Even in simpler organisms the photosynthetic pigments appear to be confined to particulate structures rather than being dispersed. All of the chlorophyll and carotenoids of the blue-green alga *Synechococcus cedrorum* are confined to particles approximately 2200 Å in diameter, roughly the size of the grana of higher plants [Calvin & Lynch (72)]. Similarly, the pigments of the photosynthetic bacterium *Rhodospirillum rubrum* were found to be in chromatophores 400 to 600 Å in diameter [Pardee *et al.* (73); Schachman *et al.* (74)].

The lamellae in the grana of higher plants may be readily separated by ultrasonic treatment, and appear to have a thickness of 70 Å (63, 64). It has been suggested that grana are composed of discs of protein material held together by intervening fatty layers. Pretreatment of chloroplasts with enzymes and fat solvents indicated that both the chloroplasts and the individual grana are surrounded by protein-lipoid membranes with an outer lipoid layer. This may be responsible for the wax-like properties of isolated chloroplast fragments and the selective staining of grana by lipophilic dyes. Different parts of the chloroplast stroma show a different electron scattering power [Bustraan *et al.* (75)]. Leyon (76) examined shadow-cast chloroplasts, chloroplast fragments, and sections of chloroplasts of *Beta sacharifera* and *Aspidistra*. The grana were found to be composed of stacks of 15 to 60 lamellae with the long axes of the stacks (which are roughly parallel to one another) arranged normal to the long axis of the chloroplast as found by other workers. He made the important observation that some lamellae appeared to continue from one stack to another so that the grana are not completely isolated from each other. However, he was unable to demonstrate the presence of membranes around either the chloroplasts or the grana. He further examined the effect of assimilate on the apparent structure of the chloroplasts. Levon (77) also examined chloroplasts from young leaves with the electron microscope. The first ultrastructures to appear in the developing proplastids were a few extended lamellae. Leyon regards this as the "primitive" condition since it occurs in protozoans and algae. He suggests that the grana are formed during later stages in the development of plastids of higher plants, and postulates that they might result from a fragmentation of the primitive structure by the accumulation of assimilate. The chloroplasts of tobacco, as described recently by Cohen & Bowler (78), present a very interesting picture in that they seem to combine the characteristics of

the flagellate and algal chloroplast with its extended lamellae and the higher plant chloroplast with its grana structure. Whole chloroplasts were carefully isolated and washed in pH7 buffer. They were then gradually dehydrated with ethyl alcohol before being embedded in n-butyl methacrylate for sectioning. The electron micrographs showed saucer-shaped chloroplasts with alternate light and dark lamellae parallel to the broad curved faces of the chloroplasts. These apparently completely traversed the chloroplast in a manner similar to those of flagellate chloroplasts (70). The dark lamellae were 140 to 280 Å thick, and the light lamellae ranged from 70 to 350 Å. The grana were disc-shaped and were also composed of alternate light and dark lamellae about 70 Å in thickness similar to those described by Steinmann (63, 64). The chloroplast stroma contained characteristic spherical inclusions of low electron density together with a few smaller spherical inclusions of high electron density. The preparatory technique apparently destroys most of the chloroplast membrane. The origin of chloroplasts was extensively investigated with the light microscope by Hertz & Maly (79). They confirmed the lamellar structure of grana, but they reported that the young chloroplasts were at first homogeneous, and that the differentiation into grana and stroma followed as a secondary process. The literature on chloroplast inheritance has been reviewed by Weier & Stocking (67).

Finean et al. (65) studied the fine structure of Aspidistra chloroplasts with both the electron microscope and with x-ray diffraction techniques. Specimens fixed in osmic acid gave better results than fresh material. The x-ray studies indicated a structure consisting of units repeating at 250 Å which was in exact agreement with the spacings of the lamellae as shown in the electron micrographs. Peripheral nerve myelin and retinal rods showed a similar correspondence between x-ray diffraction studies and electron micrograph studies.

Composition of chloroplasts.—Little, if any, precise data are available concerning the chemical composition of chloroplasts since it is extremely difficult to prepare chloroplast fragments free from contamination by other components of the cell [Wildman & Jagendorf (80); Weier & Stocking (67, 81); McClendon (82, 83); Weier (84)]. Further, it is always possible that soluble materials would be removed from the chloroplasts by the isolation medium (80). As a result, most of the information in the literature concerning the presence and distribution of proteins, enzymes, nucleic acids, and other compounds of metabolic and structural importance in chloroplasts must be examined with caution.

The literature on the protein composition of chloroplast fragments has recently been reviewed in detail by Wildman & Jagendorf (80), and relatively little new work has appeared since. Sisakyan et al. (85) examined the properties of proteins extracted from sugar beet chloroplasts by 70 to 80 per cent alcohol at pH 3.0 to 3.3 and by butanol solutions at pH 8.0 to 8.5. Sisakyan et al. (86) also prepared a protein fraction from sugar beet chloro-

plasts by the precipitation of an ethanol extract of chloroplasts with acetone. The material prepared in this manner was not electrophoretically homogeneous. The amino acid composition of the material upon hydrolysis was determined by paper chromatography and compared with that of sugar beet leucoplast protein. Andreeva & Plyshevskaya (87) raised corn and tobacco plants in a low-nitrogen medium. Ammonium sulfate containing N^{15} was then added to the culture medium and the proteins of the plastids and cytoplasm were examined for isotopic nitrogen after additional periods of growth under a variety of conditions. N^{15} was incorporated into the cytoplasmic proteins under all the conditions employed: in light and in dark, with and without carbon dioxide. On the other hand, N^{15} was incorporated into the chloroplast proteins only in the light with carbon dioxide present. The incorporation of radioactive carbon into the chloroplasts of corn plants grown in a nutrient solution containing radioactive glutamic acid was studied by Roux & Husson (88), while the effect of nitrogen nutrition and illumination on the chemical composition of chloroplasts was investigated by Osipova (89). Nezgovorova (90) found that the rate of decomposition of chlorophyll in isolated chloroplasts by various antiseptics was decreased by cytoplasmic proteins. Schieler et al. (91) grew Chlorella in a C^{14}-containing culture medium. The cells were dried, extracted and hydrolyzed with acid. Eighteen amino acids were isolated and the specific activities were determined. Differences in activity were observed which ranged from less than 2 counts/min. per mg. for proline, isoleucine, and valine to over 1000 counts/min. per mg. for phenylalanine and aspartic acid. These results were interpreted as indicating that the carbon of carbon dioxide and glucose might be incorporated into amino acids by different pathways. Yemm & Folkes (92) examined the amino acids of cytoplasmic and chloroplastic proteins of barley. Microbiological assay techniques were used. Quantitative determinations of 18 amino acids in cytoplasmic protein preparations and in mixed cytoplasmic protein-chloroplast preparations from mature leaves gave essentially identical results except for lysine.

Metzner (93, 94) examined the chloroplasts of Elodea, Agapanthus umbellatus, and Ambulia heterophylla for the occurrence and distribution of nucleic acids by standard cytochemical techniques. He concluded that the stroma contained only ribonucleic acid while the grana contained both ribonucleic acid and desoxyribonucleic acid. He also examined the effects of proteolytic enzymes on chloroplasts and found that they were quickly destroyed by alkaline trypsin and little affected by acid (HCl) pepsin. Sisakyan & Chernyak (95) examined sugar beets chloroplasts for nucleic acids. Most of the nucleic acid was of the ribose type, although traces of the desoxyribose type were present in granules in the chloroplasts. Jagendorf & Wildman (96) recently reported that carefully washed isolated chloroplasts from tobacco contained only very little nucleic acid, part of which, at least, was DNA. They also presented quantitative data on the nitrogen, chlorophyll, phos-

phorus, iron, nucleic acid phosphorus, and nucleic acid nitrogen in these purified chloroplasts.

Barley chloroplasts were examined by Davenport (97) and found to be rich in cytochrome-f and very low in cytochrome-c. The possible participation of the cytochromes in the photosynthetic process has been thoroughly discussed in a recent review by Hill & Hartree (98). Godnev *et al.* (99) used P^{32} in studying the distribution of phosphorus in the chloroplasts and other cellular structures of the leaves of oats, wheat, and lettuce. The lipoid phosphorus represented only 3.0 to 3.3 per cent of the total chloroplast phosphorus. Ratios of phospholipid to chlorophyll were calculated for the chloroplasts of the different plant species.

Pringsheim & Pringsheim (100) grew *Euglena gracilis* at temperatures just below the maximum for multiplication (34 to 35° C.). In this way, the chromatophores of most strains are caused to disintegrate. Permanently chromatophore-free races may be obtained from some strains. Short exposure to high temperatures did not prove effective in producing the above phenomenon. The eye spot remained in most colorless strains as long as they were kept in the light. In the dark the eye spot disappeared. Chromatophores and eye spots never reappear; however, the heterotrophic nutrition and reproductive vigor are not altered in the colorless strains. The high-temperature effect is similar to that produced by streptomycin [see Provasoli *et al.* (101) for a review of this phenomenon] in that those strains most sensitive to heat are also most sensitive to streptomycin. Heinrich *et al.* (102) used white *Euglena* mutants obtained with streptomycin for a simplified vitamin B_{12} bioassay technique.

A number of compounds have been recently shown to induce albinism in higher plants. Hamner & Tukey (103) found that 3-(alpha-imino-ethyl)-5-methyl tetronic acid would interfere with chlorophyll formation in certain plants. Ready *et al.* (104) showed that 3-nitro-4-hydroxybenzoic acid and other substituted benzoic acids could induce albinism in immature leaves of oat seedlings. These compounds had no effect on the plastid pigments of mature tissues. Rhykerd *et al.* (105) found that bisthiocarbamyl hydrazine, 5-aminotetrazole, and 1,2-diacetyl-3,5-diamino-1,2,3,5-tetrahydro-1,2,4-thiadiazole induced albinism in corn and soybean leaves, even in mature tissues. The effect of 5-aminotetrazole was temporary, whereas, that due to the other two compounds persisted. Bisthiocarbamyl hydrazine in the light caused a disappearance of the green color in fresh chlorophyll solutions. This compound also influenced chloroplast development.

Enzymes in chloroplasts. The literature on enzymes in chloroplasts has been recently reviewed by Weier & Stocking (67), while a survey of enzymes possibly involved in photosynthesis has recently been made by Brown & Frenkel (8). As mentioned in the preceding section, it is extremely difficult to obtain chloroplast preparations known to be free of contamination by enzymes from other regions of the cell. Thus, most reports on the enzyme

activities of chloroplasts must be accepted with reservations. Hagen & Jones (106) discussed some of the factors involved in the apparent intracellular localization of enzymes in leaf preparations including the temperature, ionic strength, osmotic pressure, and pH of the suspending medium. They examined the effect of pH on the distribution of catalase among the cell fractions of wheat leaves blended in a sucrose medium. At pH 3.3 and 5.6 most of the catalase was in the soluble fraction, while at pH 5.0 the catalase activity was all associated with the plastids, apparently by precipitation on the chloroplast materials.

Jagendorf & Wildman (96) have shown recently that carefully washed tobacco chloroplasts have little or no catalase or cytochrome oxidase activity even though they retain full Hill reaction activity. The catalase activity in tobacco leaf preparations appears to be associated with small particles resembling mitochondria which were rich in nucleic acid. Careful studies of this type should go far toward improving our knowledge of the localization of enzymes in leaf cells. Hagen et al. (107) examined the sorption of ions by isolated wheat chloroplasts and found that iron was bound very tightly, that zinc, calcium, manganese, and cobalt were bound weakly, and that there was very little sorption of potassium, rubidium, carbonate, and phosphate. Although starch synthesis in living plant cells apparently occurs in the plastids, Stocking (108) could not demonstrate the presence of phosphorylase in chloroplasts of potato, sunflower, and tobacco plants. Addition of glucose-1-phosphate to sections of starch-free leaf cells resulted in the synthesis of a polysaccharide. However, this took place in the cytoplasm outside of the plastids. Somewhat different results were obtained by Paech & Krech (109) who floated tissues of several different plants on solutions containing glucose, glucose and phosphate, and glucose-1-phosphate. They claimed that starch granules formed in approximately 6 hr. Since these were always near to or attached to the chloroplasts they concluded that the phosphorylase necessary to form starch was associated with the plastids. Tolbert [Tolbert & Cohan (110)] continued studies on the glycolic acid metabolism in green plants. Washed chloroplasts from barley contained about 10 per cent of the glycolic acid oxidizing enzyme, while the remainder was in the cytoplasm. The activity associated with the chloroplasts remained constant over the range pH 4 to 8 and was not affected by successive washings. This was taken as presumptive evidence that the activity of the chloroplast fragments in this reaction was not due to adsorbed enzyme.

Macdowall (111) found that the acid phosphatase activity of iris and spinach leaves was not associated with the chloroplasts. Jones & Hamner (112) re-examined the question of ascorbic acid distribution in leaf tissue. Studies on turnip leaves indicated that the concentration of ascorbic acid was the same in chloroplasts as in the rest of the cell. Although lecithinase activity has been reported in a number of plants, Kates (113) was the first to demonstrate that the lecithinase activity of leaves (spinach, sugar beet,

cabbage) is associated entirely with the chloroplast fraction. The leaf cytoplasm fraction was actually inhibitory to the lecithinase. The lecithinase activity of chloroplasts is markedly activated by diethyl ether with the maximum activity being attained at ether-saturation. Ether-extracted chloroplasts still required ether for activation, and none of the lecithinase appeared in either the aqueous or the ether phase of the extract. Dreft and acetone caused some activation of the chloroplast lecithinase, while *n*-butyl ether, petroleum ether, methanol, butanol, chloroform, and dioxane were without effect. Takashima (114) reported an interesting light-inhibition of the phosphatase activity of spinach leaf homogenate. The hydrolysis of glycerophosphatase by such preparations was inhibited by light while the hydrolysis of adenosinetriphosphate (ATP), pyrophosphate, and phosphoglycerate was not affected. The effect did not occur in the absence of chloroplasts. Clendenning *et al.* (115) showed that, althou͟h oxalsuccinic carboxylase is abundant in sugar beet leaves, it could not be demonstrated in the choroplast fraction. It would appear that most of the known plant carboxylases are soluble and thus appear in the supernatant cytoplasm fraction on centrifugation of macerated plant tissues. Arnon (116) found the glyceraldehyde phosphate dehydrogenase of green leaves to be localized in the cytoplasmic fluid rather than in the chloroplasts. Appleman (117) examined the chlorophyll-catalase relationship in barley seedlings under a number of conditions and concluded that a dynamic equilibrium existed between the various porphyrin-proteins in the chloroplast. He suggested that if rapid chlorophyll synthesis takes place catalase activity decreases, while catalase activity rises when chlorophyll synthesis is blocked. Woods *et al.* (118) showed that the reduction of neotetrazolium in leaf-sections of tobacco occurred primarily in the chloroplasts. Mutant chloroplasts had a much lower reducing capacity than the normal plastids. Ziegler (119) observed the reduction of triphenyltetrazolium chloride in chloroplasts. Dyar (120) found that blue tetrazolium was reduced in localized grana-like regions in the chloroplasts of higher plants. This reaction occurred only in the light and may represent a localized Hill reaction, although oxygen evolution was not demonstrated. A similar photochemical reduction was observed in the cytoplasm of a blue-green algae and in the chloroplasts of a number of other plants including bryophytes and various green, red, brown, and yellow algae.

Chlorophyll-protein associations.—It is well known that naturally occurring metalloporphyrins can combine with proteins (*a*) through primary bonds to pyrrole substituents and (*b*) by coordination at the metal atom. Numerous attempts have been made to find analogous chlorophyll-protein complexes [see Schwarze (121)]. It is obvious, however, from the ease with which chlorophyll can be extracted from plant materials by organic solvents, that primary bonds are not involved in any such analogues. Chlorophyll can, of course, associate with proteins. Many artificial association products have been described in the literature including the recent work of Zirm *et al.*,

(122), Sapozhnikov (123), and Rodrigo (124). Interpretation of the spectra and of the fluorescence properties of such complexes is not possible until more information becomes available on the state of chlorophyll aggregation in the plant and its relation to the sheet crystal chlorophylls recently described by the Rabinowitch group (see next section on energy transfer mechanisms for details). The phytyl chain of chlorophyll could probably associate with lipoprotein, but this should not influence the electronic properties of chlorophyll. Proteins might act as fluorescence activators by reacting with the magnesium atoms and in this way increase chlorophyll fluorescence without changing the relative positions of spectral peaks. Other types of interactions are unlikely since they would produce detectable changes in the pigment properties and spectra. It is conceivable that the protein could provide groups able to react with chlorophyll in one of its metastable excited states. These groups might act, through their constant presence, as a successful competitor for oxygen.

The "chlorophyll-lipo-protein" of Takashima (125) has been crystallized in this laboratory from thirty species of plant. It can be isolated from highly washed chloroplast fragments (126). Microscopically, the crystals appear to show an inhibited type of growth. This may result from surface adsorption of chlorophyll, since it appears that chlorophyll can be washed from the crystals to leave a clear crystalline material with a low melting point. The material gives all of the usual tests for protein, and, according to Takashima, it contains a large fraction of benzene-soluble material. For these reasons, the material is of interest as a possible crystalline lipoprotein.

A few unsubstantiated reports have appeared in the past concerning the preparation of artificial chlorophyll-protein complexes with photochemical activity [reviewed in (40)]. Vishniac (126a), however, has recently reported on some very significant experiments in which he found that colorless extracts of acetone powders of chloroplasts combined with a small amount of chlorophyll (as a methanol solution) could carry out the photochemical reduction of TPN as measured by the coupled reduction of glutathione. It is to be hoped that more investigations of this type will be carried out in the future.

Smith *et al.* (127) demonstrated that the Hill reaction rate of isolated chloroplasts from etiolated barley during successive stages of greening, paralleled the conversion of protochlorophyll to chlorophyll. Within experimental error, the ratio of relative Hill reaction activity to relative chlorophyll content of the chloroplasts was a constant. This was true even though the relative chlorophyll content increased by a factor of over a hundred during the course of the experiments. A protein may well be involved in the protochlorophyll to chlorophyll conversion since the system is denatured at 39°C., a temperature close to that at which thermal denaturation occurs in the Hill reaction system (see the following section on the Hill reaction). The reaction proceeds with measurable velocity down to temperatures of

−195°C. and demonstrates generally the very small temperature coefficients characteristic of photochemical reactions [Smith (128)]. In view of the very low mobility of all molecules in the frozen state at −195°C., and the poor possibilities for energy transfer at the low concentrations of protochlorophyll involved, it is surprising to find that the reaction is first order in light intensity but second order in protochlorophyll concentration. This order can not readily be attributed to pairwise dimerization or to other interactions. Smith observed that the first chlorophyll produced in fully etiolated leaves would not support oxygen evolution. It is of interest to compare this observation with the results of Clendenning & Gorham (129) who found that chloroplasts from the young leaves of many plants had a very low activity in the Hill reaction per given amount of chlorophyll as compared to the chloroplasts of mature leaves. Also Fujimura *et al.* (255) examined the photochemical activity of chloroplasts from wheat as a function of the age of the plants. Chloroplasts from young plants showed very little activity. Activity increased rapidly as the plants matured and was constant from the time of heading through flowering, and did not decline until the leaves had started to yellow. Introduction of a dark period during greening of etiolated plants greatly accelerates the gain in photosynthetic activity (128) which may indicate the need for the production of some other component to support photosynthesis. In some experiments chlorophyll-*b* was entirely absent, yet photosynthesis occurred. Krasnovskii & Kosobutskaya made similar studies (130). According to them, the first chlorophyll produced from protochlorophyll is in a "monomeric" form which later aggregates. The two forms are in equilibrium and have differently placed red peaks. The monomeric form with a peak at 670 mμ is unstable photochemically. Orientation in the grana gives the stable form absorbing at 678 mμ. The authors suggest that both forms are photosynthetically active, although this fact would be surprising if the monomeric forms consist of chlorophyll which has not as yet been incorporated into the cooperating system of pigments. Perhaps they function only as relay stations in the energy transfer system. This type of investigation looks very promising. The same authors also observed spectral changes in crystals of "chlorophyll-lipo-protein" on heating. The crystals did not fluoresce at low temperatures at which their red peak was at 690 mμ. On heating, fluorescence appeared as the peak shifted to 670 mμ. Our experience suggests that the crystals may have melted at the upper temperatures. The protein phycoerythrin from *Callithamnion ribosum* was studied by Krasnovskii *et al.* (131) by means of physical and chemical methods. It was found to consist of two components with different molecular weights. The material was not active in the Hill reaction. Nagai (131a) showed that the increased ability of the chloroplasts of etiolated plants to reduce silver salts paralleled the increase in chlorophyll formation on exposure to light.

Miscellaneous.—The biosynthesis of chlorophyll has recently been reviewed by Granick (132, 133). However, a few papers have appeared in the

past year which should be mentioned. Della Rosa *et al.* (134) examined the synthesis of chlorophyll by *Chlorella* in the presence of labeled glycine and acetic acid. The carbon atoms of acetate and the α-carbon atom of glycine were used in the synthesis of the dihydrotetrapyrrole structure of chlorophyll. This is similar to the mechanism of synthesis of protoporphyrin IX in animals. A striking difference was found, however, in that the carboxyl carbon atom of glycine is used for tetrapyrrole synthesis in *Chlorella*, whereas it is not used in animals. Isotope studies indicated that chlorophylls *a* and *b* are not interconvertible in *Chlorella*. Granick and his coworkers continued studies on the biosynthesis of chlorophyll [Granick *et al.* (135); Bogorad *et al.* (136); Granick *et al.* (137)]. Egle (138) reported on some recent studies on chlorophyll biosynthesis.

A series of recent papers has appeared on techniques for the separation of pigments and other molecules of interest in photosynthesis studies. A few of the most significant of these papers are listed (139 to 145) even though space is not available to discuss them. Evstigneev (146) reported on some critical measurements of the effect of different solvents on the absorption spectrum of chlorophyll, while Evstigneev & Gavrilova (147) examined the spectral properties of the reduced forms of chlorophyll-*a* and *b*.

A number of workers have commented on the apparent association of plant viruses whth chloroplasts [see (148) for references]. Leyon (148) made electron micrographs of chloroplasts from *Beta sacharifera* and *Chenopodium foliosum* infected with beet yellows virus. Characteristic threads of the virus were found protruding from the chloroplasts in such a way that it appeared they had been formed there. This association, which seemed to be with the stroma of the plastid rather than the grana, was very firm, which might account for the difficulties met in attempting to purify this virus. Similar studies were carried out with tobacco mosaic virus, potato X virus, potato Y virus, *Pisum* virus, and *Brassica* mosaic virus. These viruses seemed less firmly associated with the chloroplasts. This may be related to the report by Jagendorf *et al.* (148a) that the rate of turnover of N^{15} in the chloroplasts of tobacco seems to be unaffected by tobacco mosaic virus infection. It would be of interest in this respect to examine the nitrogen turnover in chloroplasts of plants infected with beet yellows virus.

It was shown a number of years ago that the infectivity of tobacco mosaic virus was markedly reduced by treating the virus with crude extracts of the leaves of higher plants. Chiba & Tominaga (149) recently re-examined this phenomenon and found that in most of the plants studied the virus inhibition activity was associated with the chloroplasts rather than with any of the other cellular components. The effect is dependent on pH and shows a temperature optimum at 35 to 45°C.

It is commonly accepted that chloroplasts do not occur in the epidermal cells of mesophytic leaves. Alscher & Lavin (150), however, recently reported finding greenish granules in the epidermal cells of spinach, which re-

semble the grana of chloroplasts of higher plants. A few papers appeared concerning the factors affecting chlorophyll synthesis and loss under field conditions (151, 152). Bandurski *et al.* (153) examined the effects of different day and night temperatures on the properties of tomato leaves. Low photo-temperatures markedly reduced the concentrations of leaf pigments, with 4°C. being physiologically equivalent to complete darkness. Godnev & Shlyk (154) reviewed the evidence for the utilization of sugars by higher plants as the raw material for leaf pigment synthesis. Roux & Husson (155) examined the incorporation of radioactive carbon into the leaf pigments of plants photosynthesizing for short periods in an atmosphere containing $C^{14}O_2$. They concluded that both chlorophylls, and especially carotenes, were rapidly destroyed and reformed. Harvey (156) examined the synthesis of chlorophyll and other pigments in the marine diatom *Nitzschia* as a function of nitrogen supply, while Finkle & Appleman (157) examined the effect of magnesium concentration on the development of chlorophyll and catalase in *Chlorella*. Whitaker (158) examined the leaf pigments of a yellow-green muskmelon mutant. The role played by chlorophyll in plant transpiration was studied with the aid of new techniques by Sivadjian (159). Shaw & MacLachlan (160) examined the chlorophyll content and carbon dioxide uptake of stomatal cells.

Energy Transfer Mechanisms

Pigment interactions and the migration of energy.—Electronic quanta can be transferred from molecule to molecule by mutual coupling of dipole fields in a process known as "sensitized fluorescence" or "induced resonance." The collection of photons at the initial chemical reaction sites in vision probably proceeds by such a process, as may the photosensitization of photographic emulsions by dyes (161). Dutton *et al.* (162, 163) first obtained evidence that energy transfers of this type were involved in photosynthesis, and the process has since been widely studied in this connection. Recent investigations of energy transfer, or relative photosynthetic efficiency of pigments in plants, include those by Tanada (164), Thomas (165), and French & Young (166). The most complete study is that of Duysens (167, 168), who systematically measured energy transfer in purple bacteria, diatoms, and in green, brown, blue-green, and red algae. By making relative fluorescence measurements, he showed that electronic excitation energy is transferred by a "bucket brigade" of pigments from those absorbing toward the blue end of the spectrum to those absorbing at longer wave lengths until chlorophyll-*a* or the form of bacteriochlorophyll absorbing at 890 mμ was reached. Useful internal conversion apparently occurs only at these latter pigments or at chlorophyll-*d* when present since these alone show fluorescence. Generally speaking, carotenoids are approximately 50 per cent efficient in energy transfer, although fucoxanthol (164), which fluoresces farther toward the red than the others, has a much higher efficiency. Phycobilins,

phycoerythrins, and chlorophyll-*b* are nearly 100 per cent efficient in transfer and in causing photosynthesis. Migration proceeds in one direction as might be expected if the migration time is longer than vibrational periods (10^{-13} sec.) In each step toward the red the electronic quantum of the emitter is converted into a lower energy electronic quantum of the absorber together with vibrational quanta which are dissipated before the reverse transfer process can occur.

The red alga *Porphyridium cruentum* and the blue-green alga *Oscillatoria* contain quantities of nonfluorescing chlorophyll-*a* which cannot participate in photosynthesis (168), possibly because it can lose its energy noneffectively to traces of chlorophyll-*d*. Phycocyanin energy migrates only to the fluorescent chlorophyll-*a*, and in this way phycocyanin appears more efficient per molecule than chlorophyll-*a*. These facts and interpretations probably explain the finding of just such a phenomenon by Haxo & Blinks (169). Duysens (168) assumed that energy absorbed in the blue bands of chlorophyll-*a* and *b* was as efficient in fluorescence and photosynthesis as that absorbed in the red. This assumption is well supported by the findings of Forster & Livingston in pigment studies *in vitro* (24, 26).

Sensitized fluorescence may not only provide the means for energy transfer between pigment molecules but also between protein and pigment. Bücher & Kaspers (170) noted that carbon monoxide can be driven off the heme iron of hemoglobin just as efficiently by light absorbed in the protein portion of this conjugated protein as by light absorbed in the heme. Recently Bannister (171) has observed that phycocyanin fluoresces with the same intensity in light absorbed by the protein as by that absorbed by the chromophore. Since the theories for the process restrict the transmitter (tryptophan, tyrosine, or phenylalanine side chains) and the receiver (chromophore) to but a few relative orientations, it would be most remarkable if high efficiency could be demonstrated in dilute solution under which conditions the energy-transfer process would have to be intramolecular rather than intermolecular. This subject, therefore, has significance for the entire problem of protein structure and function.

There are two general theoretical approaches to the problem of transferring pure electronic energy [Bayliss (172)]. That of Fraenkel & Peierls [Seitz (173)] applies to crystals and ordered arrays so strongly interacting that the crystal takes on, at least quantum-mechanically, some of the aspects of a single molecule. The electronic energy can be thought of as being transferred by wavelike displacements of electrons from their equilibrium positions. The process bears the name "exciton" migration. A quite different process occurs when the electric fields (usually the dipole fields are most important) of an excited and an unexcited molecule interact to provide the vehicle for energy transfer. This process ("sensitized fluorescence"), which is best described in modern theoretical language by Förster (174, 175), involves a sort of controlled emission and reabsorption of light energy which

can operate with great efficiency, apparently over distances as great at 70 to 100 Å.

These two apparently divergent phenomena have turned out to be rather similar on the basis of recent theoretical studies, particularly those of Heller & Marcus (176). When one considers not just pair interaction alone, but the summed influence of all near neighbors on pair interaction, then sensitized fluorescence begins to resemble exciton migration. And when one notes that dipole interactions in crystals should be strong enough to support exciton travel, then the latter process begins to resemble sensitized fluorescence. In the latest studies on chlorophyll the two processes appear to have finally coalesced. For example, the transmission spectrum of crystals of ethyl chlorophyllide is reported by Rabinowitch et al. [(177, 178); see also Strain (179)], to shift toward the red as the size of the crystal increases. The largest shift of the red peak observed was from 645 mμ to 740 mμ. This type of property has been considered a characteristic peculiar to the strong crystals of the ionic or covalent type. It is well explained for strong crystals on the basis of electron interactions among many well-coupled atoms, and it is closely connected with the ability of a structure to support exciton migration. That this phenomenon can occur in the weak molecular type of crystal formed by ethyl chlorophyllide seems to substantiate the prediction of Heller & Marcus (176) and to support the possibility of pure exciton motion in molecular crystals of dye molecules with overlapping fluorescent and absorption spectra as required for suitable dipole interactions. Thus, pigment molecules fluorescing and absorbing at some of the same wavelengths when arrayed in any regular fashion, cannot be expected to act like so many independent molecules (175).

The spectral shift described above has also been observed in surface layers of ethyl chlorophyllide and of chlorophyll prepared on water and then lifted onto glass plates as described by Jacobs et al. (180). The red peak was at 730 mμ in both ethyl chlorophyllide and chlorophyll, whereas, a pigment layer typical of amorphous colloidal chlorophyll had its peak at 670 mμ. Aronoff could find no spectral changes in highly concentrated (0.1 M) chlorophyll solutions (181). The ethyl chlorophyllide layer formed an essentially two-dimensional crystal which was only one molecule thick. Chlorophyll-a and bacteriochlorophyll, or mixtures of chlorophylls-a and b in the ratio of 3:1 can now be crystallized from organic solutions in the presence of water by several procedures [Jacobs, Vatter & Holt (182)]. The very thin plates thus formed demonstrate red-peak shifts to as far as 770 mμ. Water is required for crystallization of the various chlorophylls and chlorophyllides but not for the pheophytins. This suggests that water associates at the magnesium and perhaps that fluorescence activators are necessary for crystallization. If so, this suggests again the presence of a net positive charge on the magnesium atoms which would produce repulsion between chlorophylls. The 3:1 ratio of the chlorophylls needed for crystallization is

also suggestive, since this is the reported ratio of their occurrence in higher plants. Might it not also be possible that carotene molecules will be found to crystallize with chlorophyll in the ratio of 1:2 which would explain the natural occurrence of the two pigments in these proportions?

The various spectral shifts described above certainly have some bearing on the positions of the spectral peaks observed in natural chlorophyll-containing systems. Krasnovskii *et al.* (183) was able to prepare solid films of bacteriochlorophyll from solutions of a single species of the molecule which showed the same three far-red peaks as found in the living bacteria. Furthermore, the electron micrographs of chloroplasts are readily interpreted as consisting of sheets of chlorophyll, probably no more than two layers deep, confined between lipoid and protenoid layers (see previous section on chloroplast structure). Wolken & Schwertz (71) have proposed a model for chloroplast structure with these characteristics on the basis of their careful measurements of the amount and distribution of chlorophyll, the position of which appears to be clearly distinguished in electron micrographs by the high electron density of the chlorophyll aggregates. It is intriguing to compare this structure with the sheet crystals of chlorophyll described above. However, two-dimensional crystallization is probably not very selective and would permit the formation of many types of mixed crystals, only a very few of which would be likely to have the necessary properties to support photosynthesis. In any event, the spectral shifts in plants are smaller than the extremes noted in sheet crystals by the various workers, so we may assume that the natural layers have less extensive integrity. Nor can we discount possible spectral changes due to (*a*) compound formation between pigments and protein or lipoid, or (*b*) physical adsorption effects due to weak interaction with associated plant materials. Either (*a*) or (*b*) might include the possibility that association stabilizes some tautomeric form of chlorophyll with which we are not yet familiar. Possibility (*a*) is probably disallowed by the fact that no pronounced changes in the relative positions of the major peaks of chlorophyll exist between chlorophyll solutions and chlorophyll in the plant. The phytyl tail plays no part in establishing the visible spectrum of chlorophyll [Watson (184); Holt & Jacobs (185)]. It probably functions merely to position the chlorophyll and to provide a mechanism whereby newly formed chlorophyll molecules can secure positions in the existing array. One may speculate about the possibility that these hydrocarbon tails tie carotene molecules to chlorophyll to provide the close association so frequently noted when these pigments are isolated from plant materials. Interactions of plant substances with the tail alone cannot influence the spectrum in any fashion, but any associations with the tetrapyrrole should alter the spectrum. We have noticed, however, that this statement cannot apply to interactions with the magnesium atom.

Layer-like crystallization of chlorophyll in the plant would be expected to have a number of effects on the utilization of light energy. The resonance

transfer of electronic quanta should be an especially good process with the pigment in this form. Fluorescence should be greatly diminished, partly because of efficient reabsorption of fluorescent light, and partly because of the intrinsic characteristics of such crystals [Kallman & Furst (186, 187, 188)]. Both of these properties are exhibited by chlorophyll *in vivo*. Duysens' value for the *in vivo* fluorescent yield of 1 per cent is some tenfold higher than the older values, but even this value still represents but 4 per cent of the best values secured from *in vitro* systems (168). It is the opinion of Jacobs, Holt & Rabinowitch (180) that their sheet crystals of strongly interacting chlorophyll molecules do not occur *in vivo* as judged by the spectrum of plant chlorophyll. It seems doubtful that this argument can rule out crystallites containing a few hundred or a few thousand chlorophyll molecules. In any event, the sheet crystals of chlorophyll described by the Rabinowitch group appear to be just the proper materials for designing a photochemical system to permit the best utilization of light.

The Hill Reaction

Isolated chloroplast preparations from algae and higher plants, as well as intact algae, have the ability to carry out the step in photosynthesis most difficult from an energetic consideration, the splitting of water. As Hill first showed (189), these preparations in the presence of a suitable electron acceptor can carry out the oxidation of water to hydrogen ions and oxygen gas on illumination. This process apparently represents the light-absorbing and water-splitting reactions of the overall photosynthetic process (2). The chloroplast system is much simpler than that required for photosynthesis, and it may be easily controlled to give precise and reproducible data. It would seem, therefore, that the Hill reaction offers an ideal means for attacking the problem of photosynthesis at the light end of the reaction. In view of its importance, it is surprising that so little work is being carried out on this reaction at present.

Components of the Hill reaction.—Two groups of workers have recently obtained the Hill reaction with call-free preparations of green algae [Hill *et al.* (190); Punnett & Fabiyi (191)]. McClendon & Blinks (192) were similarly successful with chloroplasts from red algae. In this case it was necessary to use high molecular weight solutes such as polyethylene glycols to obtain active preparations. These materials preserve the normal morphology of the chloroplasts, perhaps by providing a more normal osmotic environment. In spite of these successes, most Hill reaction studies are carried out with whole algae or with isolated chloroplast preparations from higher plants. The latter have many advantages, and they can be prepared in such a way as to permit Hill reaction rates comparable to the photosynthesis rates of the intact leaves of the same plant.

There is little definite information concerning the chemical nature of those chloroplast components involved in the Hill reaction. Soluble organic

components are apparently not involved, since extensive washing or dialysis, per se, of chloroplast preparations does not decrease their activity (see section on effects of extrinsic factors for a discussion of the possible requirements for certain inorganic ions). Wessels, however, has reported an effect due to washing (193, 194, 195). The dark steps in the Hill reaction apparently depend on some protein component, since this part of the process can be retarded by ultraviolet radiation [Holt *et al.* (196)] and by thermal inactivation in reactions characteristic of proteins (197, 198). The protein involved in thermal inactivation might be Franck's enzymes B or C, part of the pigment supporting structure, or even part of a chlorophyll-protein complex. In the latter event the spectrum of the chloroplast material might be expected to change with thermal denaturation. No such behavior has been noted [Bishop *et al.* (197), but see (130)]. The kinetics of the thermal inactivation of chloroplast preparations from sugar beet and chard appears to involve two processes. One of these is significant only at relatively high temperatures (above 30°C.) and appears to be first order. The other is significant at both high and low temperatures and is second order. Thermal studies on chloroplasts are complicated by an activation phenomenon [Bishop *et al.* (197)]. Chlorophyll is almost certainly involved in the slowest step of the photochemical part of the Hill reaction. Otherwise it would be difficult to explain the very high photochemical efficiency of photosynthesis (see section on quantum requirements).

Formal mechanism of the Hill reaction.—The study of the mechanism of the Hill reaction by a kinetic approach has been greatly simplified by the finding [Lumry *et al.* (1, 199)] that washed chloroplast fragments from a number of plants show a Hill reaction velocity-light intensity relation which strictly follows a simple rectangular hyperbola even up to very high light intensities (2×10^4 ergs/cm.^{2} at 675 mμ). This relationship may be expressed by the following equation (199):

$$v = \frac{k_d I}{I + K} \qquad\qquad 1$$

where v is the Hill reaction velocity and I is the light intensity. This equation, which is similar in form to the Briggs-Haldane representation of the Michaelis-Menten equation used in studies on enzymes, has two parameters, a scale factor k_d and a fitting parameter K. Since k_d and K have the same temperature coefficient, K may be considered to be compounded of k_d and another factor, k_L, with $K = k_d/k_L$ (2). The factor k_L, which is temperature independent, is associated with the light step of the Hill reaction, while k_d, which has an activation energy of 10 kcal. (197), is a measure of the limiting dark reaction or Blackman phase. Equation 1 cannot be used in this simple form when the reaction system absorbs more than about 30 per cent of the incident light. It can be modified for use with more strongly absorbing systems, however. An application of this equation to experimental data

permits distinguishing mathematically between the two, and only two, slow steps which appear to be involved in the Hill reaction. These rate parameters cannot be assigned to any known reactions at present. They are not pure rate constants in the usual sense, as they contain hidden concentration factors. For example, the concentration of Franck's enzyme B is apparently a factor in k_d, while chlorophyll concentration appears to be involved in k_L.

It should be possible to enumerate and characterize other hidden factors (rate constants, reactant concentrations) by careful and systematic studies of those variables which influence the rate of the Hill reaction. Inhibitors are particularly useful for such studies. We may distinguish four simple types of inhibition: k_L alone inhibited, k_d alone inhibited, k_L and k_d both inhibited in the same way, and k_L and k_d both inhibited, but in different ways. Thus far in our laboratory we have detected no substance capable of inhibiting k_d and k_L in the same way. This suggests that the reactions for which the two parameters provide effective rate constants have no reactant in common. Orthophenanthroline, for example, decreases both rate parameters, but in different ways and at different concentrations (200). Phenylurethane, on the other hand, depresses only k_d. Its effect on k_d is to give a simple mass law relation which is half-order in phenylurethane concentration (201). The indifferent narcotics, of which phenylurethane is a classical example, are supposed to act as inhibitors by being adsorbed on active catalytic surfaces. This decreases the available catalytic area and thus slows down the rate of the reaction concerned. With respect to photosynthesis, narcotics are supposed to inhibit to the same relative extent at both low and high light intensities. That is, the narcotic, by being an indifferent surface-active agent, is supposed to be adsorbed on both pigments and enzymes and thus interfere with both photochemical reactions and the succeeding dark (enzymatic) reactions. In the terminology introduced above, then, phenylurethane should inhibit both k_L and k_d. Since it inhibits only k_d it should perhaps be considered as a specific inhibitor, and not as a narcotic. Wassink & Kersten (202) found that phenylurethane inhibited the photosynthesis of the diatom *Nitzchia* to a much greater extent at high than at low light intensity, but the inhibitable step does not seem to be that normally appearing in algal photosynthesis or the Hill reaction. Such results require a reevaluation of the concept of "narcotization" in photosynthesis studies.

The above data make clear the necessity for carrying out complete velocity-light intensity experiments in studying the photochemical properties of chloroplast preparations. The resulting data should be interpreted by means of an appropriate form of Equation 1. This procedure is necessary because light saturation occurs at such low intensities in chloroplast systems. Even at the lowest light intensities which still provide reaction rates of sufficient precision, a pure k_L rate does not occur. As a result, meaningful k_L values can be obtained only by the extrapolation of velocity-light intensity curves to nearly zero light intensity.

A number of interpretations of light intensity-photosynthesis rate data

appear in the literature. Many of these do not properly account for the decrease in intensity of the light beam as it passes through the photosynthesizing system. Burk presents data on *Chlorella* photosynthesis as a function of light intensity which satisfy a rectangular hyperbolic relationship (203). Gorski (204, 205), in some recent work, has arrived at what seems to be similar conclusions concerning the relationship between light intensity and photosynthesis rate by an extension of Blackman's old "limiting factor" concept. It should be pointed out that at present there is no fundamental reason for assuming that the Hill reaction and photosynthesis should, of necessity, follow the same rate *versus* light intensity relationship.

Mode of oxidant participation.—Oxidants are required for the Hill reaction. However, their presence influences the reaction in several ways. Oxidants such as ferricyanide and quinone do not affect k_d or k_L in the concentration range 10^{-6} to 10^{-3} M. (199). This indicates that the reduction step of the reaction is either not rate limiting, or that it is not dependent on oxidant concentration over this range. At concentrations higher than 10^{-3} M., k_L decreases progressively while k_d rises, passing through a maximum at approximately 10^{-2} M., and then falls off rapidly. These effects are reversible only slowly, if at all, so that in ordinary experiments the reaction rate is not affected as the oxidant concentration changes during the course of the experiment. The rate, then, is determined by the initial oxidant concentration. The usual Warburg technique for measuring Hill reaction rates requires oxidant concentrations higher than 10^{-3} M. Thus, to be meaningful, effects studied in this way must be shown to be independent of oxidant concentration and type. These complex effects of oxidants at higher concentrations perhaps explain the many observations in the literature where experiments run at a single light intensity gave rates which were apparently a function of the nature of the oxidant used and its initial concentration [Spikes (206); Ehrmantraut & Rabinowitch (207); Brin & Krasnovskii (208), etc.]. Recently Fujimura *et al.* (256), in an extensive and very carefully documented series of experiments, reported that the Hill reaction of isolated spinach chloroplasts with ferricyanide as oxidant was zero order in ferricyanide over the range 0.5 to 10×10^{-3} M. Rate measurements were made with a potentiometric technique. This work was carried out at a constant (and relatively low) light intensity. Thus, it is difficult to compare these results with studies carried out as a function of light intensity as described above.

Wessels (193, 195), in some very recent work, examined the activity of a variety of quinones and dyes of varying standard half-cell potentials as Hill reaction oxidants. Compounds with an E_o' of approximately 40 mv. (at pH 6.5) apparently cause a reduced rate of reduction which cannot be attributed to any of the oxidant effects described above. Illuminated chloroplasts could not reduce substances with E_o' of less than 40 mv. in the presence of oxygen. Under anaerobic conditions, however, dyes with E_o' as low as -100 mv. could be reduced. Macdowall (209) also studied the "maxi-

mum" reducing power of illuminated chloroplasts with oxidants of different E_o'. As Wessels and others [Spikes *et al.* (210); Hendley & Conn (211)] have pointed out, the lowest potential which can be reached in a Hill reaction system is limited by the back oxidation of the reduced form of the oxidant. This reaction may be catalyzed by the chloroplasts. Presumably, most, if not all, of the compounds examined by Wessels were actually reduced. This would escape detection, however, if the back reaction went too readily. Even without the assumption that all were reduced, it is apparent that the Hill reaction is almost completely unspecific with respect to the chemical nature of the oxidant as long as the E_o' is above some lower limit. These results, together with the independence of rate on oxidant concentration (in the absence of secondary effects as described above), would be expected only if the natural reductant was in the excited electronic state or existed as a high energy radical in the ground state. (That is, in the latter case it would require unpaired electrons of great reactivity.) The reduction process is thus independent of the reduction potential of the oxidant so long as the latter is above the chloroplast potential. Energetic considerations, and the experiment of Uri (39) [confirmed and extended by Wessels (193)], in which illuminated chloroplasts were found unable to initiate radical polymerization, may exclude the participation of free radicals in the Hill reaction.

Vishniac & Ochoa (212) observed that TPN could function as an oxidant in the Hill reaction even though the reduced form could not be detected except by coupling to an appropriate enzyme system [also see Ochoa & Vishniac (213); Vishniac & Ochoa (214); Arnon & Heimbürger (215); and Vinogradov *et al.* (216)]. The coupling reaction was sufficiently fast to compete with back oxidation. This could be shown clearly when glutathione acted as the ultimate oxidant in the presence of a TPN-coupled glutathione reductase system [Hendley & Conn (211)]. In the latter experiments the rates of reduction were very close to those obtained with quinone as oxidant. This would indicate that the usual slow rates observed in TPN-coupled Hill reaction systems probably resulted from back reactions. Such back reactions appear to be catalyzed by the illuminated chloroplasts. It would appear then, that the illuminated chloroplast has a potential at least as low as -300 mv. (approximately the E_o' of the TPN system), and is thus able to drive many energy-requiring reactions in the plant. Evans & Nason (217), for example, recently coupled the nitrate reductase system from soybean leaves (which is DPN[4] or TPN-mediated) to chloroplast preparations and obtained a photochemical reduction of nitrate to nitrite. Vishniac & Ochoa (214) obtained the photochemical synthesis of ATP from ADP[4] in a DPN-mediated system containing chloroplast preparations and either mung bean or rat liver mitochondria. It should be noted that the redox potential of the chloroplast system may be much lower than indicated by the equilibrium potential E_o' if the reduction proceeds in two one-electron steps, one difficult and one easy; as in the production of "semi-quinone" type molecules.

It is of considerable importance to establish whether the back oxidation reactions in illuminated chloroplast systems are with oxygen or with some intermediate produced in the water-oxidation system. Mehler (218, 219) and Mehler & Brown (220) have clearly demonstrated by enzyme and mass spectrometer studies that oxygen itself is a Hill reaction oxidant. The oxygen is apparently converted to some peroxide in the reaction, perhaps hydrogen peroxide. Addition of catalase in concentrations which permit it to act as a peroxidase results in the coupled oxidation of added materials such as ethanol. The photochemical oxidation effects described in the preceding paragraph may be based on a reaction of this type. Mehler (219) observed a stimulating effect of quinone on these catalase-containing systems. This phenomenon may be related to the effect of quinone on k_d as discussed in the preceding section, and adds to the group of peculiar effects produced by quinone. It may be remembered that intact algae usually give the Hill reaction only with quinone as oxidant, following which they are unable to photosynthesize. Mehler also noted that illuminated chloroplasts could oxidize ascorbic acid in the presence of quinone in a reaction which apparently did not involve peroxides. He suggested that the quinone-ascorbate couple competed successfully with the system leading to water oxidation. Since ascorbate was oxidized, it may be supposed that hydroquinone displaced the natural water-oxidizing system in the reaction with high-energy intermediates. Similar results have been obtained recently by Vernon & Kamen with preparations from both higher plants and photosynthetic bacteria (221). In this work the water oxidation process of the usual Hill reaction was replaced by a coupled oxidation of ascorbate in the presence of cytochrome-c or 2,6-dichlorophenolindophenol. Both of these compounds, like quinone, are oxidants for the Hill reaction. Such work provides a promising new approach to the study of the oxidation phase of the Hill reaction.

Kamen and co-workers [Vernon & Kamen (223); Kamen & Vernon (224)] have investigated a photooxidation of cytochrome-c which is carried out by whole cells and sonic extracts of photosynthetic bacteria. This reaction is probably similar to the photocatalytic back reaction of Hill reaction systems as described above. Of considerable importance is the observation [Vernon (222)] that the photosynthetic bacterium $R.$ $rubrum$ contains a very high concentration of cytochrome-c, and the subsequent report [Elsden, Kamen & Vernon (225)] that the material is not identical with mammalian cytochrome-c. It has been temporarily designated a cytochrome-$c2$. Kamen's group also observed in photosynthetic bacteria a light-activated cytochrome oxidase as well as a cytochrome oxidase active in the dark. The possible relation of these enzymes to photosynthesis is not clear in view of the report by Jagendorf & Wildman (96) that tobacco chloroplasts free of cytochrome oxidase (and catalase) still had full activity in the Hill reaction. The Kamen group reported spectral changes in the course of cytochrome-c oxidation in agreement with the observations of Duysens (226) that cytochrome in purple

photosynthetic bacteria and *Chlorella* shows a spectral change on illumination in the presence of oxygen. This phenomenon should not be confused with the oxygen-independent changes in the spectrum of bacteriochlorophyll which occur on illumination in photosynthetic bacteria [Duysens (168)].

The cytochrome systems, which are known to participate in respiration, provide an attractive model for the stepwise oxidation system which seems,to be required for photosynthesis. Thus, the quantitative determination of spectral changes during the course of photosynthesis or of the Hill reaction provides a new and promising method of investigation. We do not yet know the relation between the new cytochrome-$c2$ of photosynthetic bacteria (225) and the cytochrome-f found in chloroplasts by Davenport & Hill (227). They may be identical. Cytochrome-f has a standard half-cell potential approximately midway between that of the oxygen electrode and the usual estimate for the maximum reducing power of illuminated chloroplasts. Davenport *et al.* (228) showed that a soluble protein-like factor from the plant cytoplasm was necessary to couple muscle methemoglobin as a Hill reaction oxidant to the chloroplast system. This unknown substance might also act as a transfer system between chloroplasts and the new cytochrome-$c2$. However, ordinary cytochrome-c will act as a Hill reagent without this factor [Mehler (218); Rosenberg & Ducet (229)].

Krasnovskii & Kosobutskaya (230) reported that, following a period of illumination, cell-free bean leaf preparations contained a substance which could reduce such compounds as safranine and riboflavin in the dark. This resembles the sunflower leaf preparations of Spikes *et al.* (231) which contained some material capable of reacting at an electrode which was reduced in light and oxidized in the dark. The lowest potentials obtained in this latter system, however, would not permit the reduction of safranine or riboflavin to any measurable extent. Highly washed chloroplast fragments may themselves be reduced somewhat on illumination. The light-reduced fragments could be backtitrated with ferricyanide by a mechanism which indicates the presence of two different reduced substances, one capable of reacting much more rapidly than the other [Gilmour *et al.* (232)]. At least some of the fast-reaction sites were on the surface, since the chloroplast fragments themselves would react reversibly at a platinum electrode. This indicates that Hill reaction oxidants can probably react at the surfaces of the chloroplast materials. Such surface sites could account for the ability of fragments to reduce cytochrome-c, a protein with a molecular size which is probably too large to permit diffusion into chloroplast preparations rapidly enough to permit the observed Hill reaction rates.

The possible participation of 6,8-thioctic acid (α-lipoic acid, Protogen A, pyruvic acid oxidase factor) in photosynthesis as the site of the primary quantum conversion act has been discussed extensively by Calvin and co-workers (233 to 239). This proposal is based on the following evidence. Benson & Calvin (240) and Weigl *et al.* (241) showed that recent photosyn-

thate was not incorporated into the tricarboxylic acid cycle during illumination. The only known way in which photosynthate can enter the tricarboxylic acid cycle is by the condensation of acetyl coenzyme A with oxalacetic acid. Oxidized (disulfide) thioctic acid is required for the formation of acetyl coenzyme A. The Calvin group suggested that in the light the thioctic acid is kept in the reduced (disulfhydryl) form. As a result, no acetyl coenzyme A could be formed from recent photosynthate and thus recently incorporated carbon could not enter the tricarboxylic acid cycle. It was further suggested that disulfide thioctic acid was reduced by the direct transfer of electronic excitation energy from illuminated chlorophyll. Calvin proposed that this process represents the chemical reaction in which the energy of the light quantum is converted from electromagnetic into chemical energy, i.e., the primary quantum conversion reaction in photosynthesis. The most recent formulation of this reaction is shown in Illustration D (239). The reactions with DPN and ATP are included to show how the proposed mechanism could couple to know energy transfer systems.
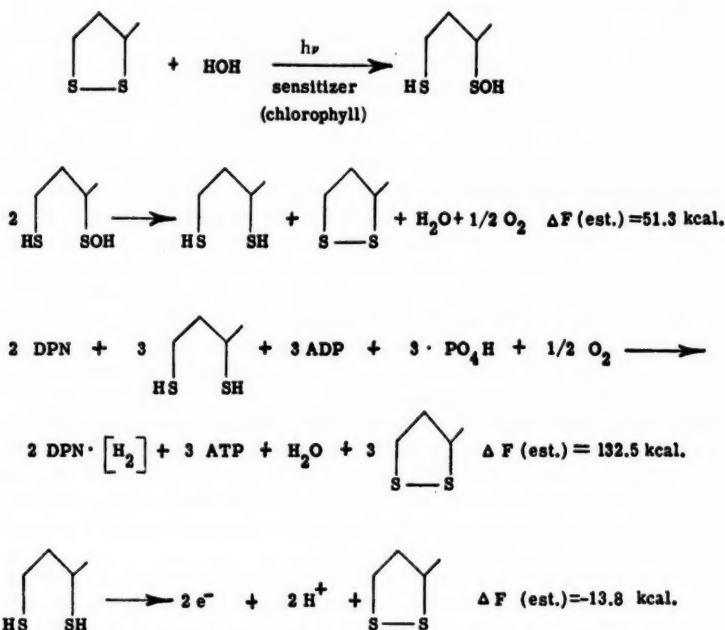
Illustration D

The mechanism as presented above is very appealing, and thioctic acid is known to occur in green leaves in considerable quantity. However, the experimental data presented to date cannot be considered to establish the hypothesis completely. The strongest support comes from the observation that pretreatment of intact *Scenedesmus* cells with 6,8-thioctic acid gives an increased rate of oxygen evolution in the Hill reaction with quinone as oxidant. Another form, the 5,8-isomer is without effect. Although the 6,8-thioctic acid did not appear to serve as a Hill reaction oxidant, it did increase the rate of the dark reaction (k_d step), but only at quinone concentrations from about 3 to 12×10^{-3} M. In this concentration range quinone also accelerated the reaction, while at higher concentrations both thioctic acid and quinone were inhibitory. The activating effect of quinone and ferricyanide in the above concentration range on the dark step of the Hill reaction as obtained with isolated chloroplasts of higher plants (199) was discussed earlier in this section of the review. On this basis, the possibility cannot be ignored that thioctic acid might activate the Hill reaction in a manner analogous to other oxidizing agents. It has been suggested that thioctic acid must be conjugated in some way, perhaps to a specific protein, in order to function in photosynthesis. In the latter case, one would not expect Calvin's experiments nor the criticisms given here to have much bearing on the question of thioctic acid participation. In any case, it is surprising that thioctic acid should have no effect on the light reaction if it is the primary energy acceptor.

Another way to examine the role of thioctic acid is to use inhibitors known to act at sulfhydryl groups. Wessels (193) has shown that *p*-chloromercuribenzoate and other sulfhydryl inhibitors have no effect on the Hill reaction. The possible participation of thioctic acid in photosynthesis has also been briefly discussed by Reed (242).

Calvin & Barltrop (233) reported on model experiments in which visible light absorbed by zinc tetraphenylporphin produced polymerization of trimethylene disulfide. Bradley & Calvin (238) studied open chain and unstrained cyclic members (such as 5,8-thioctic acid) of the thioctic acid family. They concluded that strain produced by the geometry of the five membered ring of 6,8-thioctic acid was responsible for the reduced strength of the disulfide bond in this compound. Such strained compounds show an increased reactivity. That is, the reactions in which they participate show a lower activation energy, since the strained bonds resemble the interactions between pairs of free radicals. Strained or stretched bonds may play important parts in the mechanism of action of enzymes, which, like all catalysts, act to reduce the free energy of activation of the reaction catalyzed. It may be energetically advantageous for a protein to form strained or stretched primary bonds if in so doing a number of weaker bonds can be made. These bonds could then act as the site for catalytic action. The same considerations apply to the oxidizability of sulfhydryl groups. When the reactions of such groups as, for instance, the formation of a disulfide bond, require a preliminary re-

folding of the protein to form a nonnormal state of higher free energy, the strength of the new sulfur bonds will be reduced by the amount of the configurational free energy increase [Lumry & Eyring (243)].

Of the numerous substances studied in our laboratory only the strong Hill oxidants ferricyanide and benzoquinone inhibit just the light limiting step, both reducing k_L by about 50 per cent at 5×10^{-3} M (199). The stimulating effect of these reagents, previously described, does not appear to be directly related to their inhibiting function and it is not known as yet whether these reactions are characteristic of Hill oxidants as a class. Oxygen is a poor Hill oxidant being unable to compete with ferricyanide present at concentrations several fold less than oxygen. It does, however, inhibit photosynthesis and at sufficiently high concentration displaces normal photosynthesis by photoxidation (reviewed in 40). The inhibitory action at lower pressures may be related to that demonstrated by Hill oxidants. The photoxidation reaction appears to be unique for oxygen. These observations raise some question as to the manner in which oxygen can attack chlorophyll molecules *in vivo*. While it may be a bit early to apply the results of photochemical studies of chlorophyll in organic solutions to the plant system, still, there appears very little indication that such studies are not directly applicable. As previously discussed, chlorophyll in plants does not seem to have different electronic properties from those demonstrated *in vivo* except in so far as they may depend on some peudo-crystalline arrangement in the manner described in the section on energy transfer.

Chlorophyll in plants appears to be fluorescence-activated for, if this were not the case, the excitation energy would be lost to heat internally long before it could be used in photosynthesis. Furthermore, chlorophyll must somehow be protected from oxygen for oxygen, as Livingston & Ryan (30) showed, short-circuits the transition to the metastable state and removes the excitation energy rapidly in a reaction probably related to the very efficient quenching action of oxygen on chlorophyll fluorescence. Unless the quenching by oxygen is a normal step in photosynthesis, as does not appear to be the case (see later sections), the plant must provide, through steric arrangement, a means whereby oxygen is prevented from reaching excited chlorophyll molecules. Either a very efficient competitor is available which can divert the energy to useful purposes before oxygen can dissipate it, or the chlorophyll molecule is so buried in fixed substances as to be unexposed to oxygen. Fluorescence studies of the Hill reaction provide further evidence on this matter. Zedlitz [quoted by Kautsky (244) and Shiau & Franck (61)] both noted that quinone reduces the level of fluorescence in the Hill reaction. They observed conflicting effects of oxygen. In the former study, oxygen depressed fluorescence slightly in relation to the fluorescence in nitrogen; in the latter, it stimulated steady state fluorescence by about 20 per cent. Since oxygen was present at pressures sufficient for at least 20 per cent quenching had chlorophyll been in a state equivalent to that in organic solu-

tion (401), it again appears that chlorophyll in plants must be protected from oxygen.

Kautsky (244) has suggested that some special material is present in plants to pick up the energy from chlorophyll and transfer it into the chemical system of photosynthesis. Any changes in fluorescence of the pigment is supposed to be mediated through this substance. This idea is very attractive for it provides an energy pickup process which can take place without chemical reaction of chlorophyll. We have pointed out that the *in vitro* photoreactions of chlorophyll are inefficient except when oxygen is involved. This inefficiency may be mitigated in plants by providing a substance similar to oxygen and able to compete successfully with oxygen and other Hill oxidants. It is this type of mechanism which is suggested by the studies of of Schenck discussed in the first section. Oxidants would then enter the Hill reaction by reacting with this special pick-up substance or another intermediate farther along the reaction chain. Their influence on fluorescence *in vivo* could then result from rapid reaction with the pick-up substances to make them available for further reaction with excited chlorophyll molecules. We may suspect that the postulated pick-up substance, like oxygen, can induce crossing out of the excited singlet state of the pigment and thus quench its fluorescence. A picture such as this can be tested by more experiments on fluorescence intensity and fluorescence yield in the Hill reaction. Such studies are also in need of extension as a result of the findings of Mehler, Vernon and Kamen, and others described in the previous section, but especially in the light of the report by the Krasnovskii group that chlorophyll exists in at least two different active forms *in vivo* (130).

A further possible source of information about the system of substances through which light energy is converted to chemical energy is provided by the interesting chemiluminescence reaction studied by Strehler & Arnold (245, 246). The chemiluminescence is of very low intensity varying in a way which closely conforms to the relative velocity of the Hill reaction occurring at the same time. *Chlorella* and spinach chloroplast fragments both demonstrate the reaction. The emission spectrum is that of chlorophyll indicating that part of the light energy originally imparted to pick-up substances by chlorophyll can be returned to it. Furthermore, these pick-up substances must have a very long life time for chemiluminescence continues for many minutes after illumination has been stopped. Carbon dioxide decreases the intensity of luminescence in *Chlorella* during photosynthesis so that the energy storage substances have apparently not undergone oxidation. Strehler and Arnold provide abundant evidence that high energy forms of chlorophyll-*a* are not the storage intermediates and there is very little evidence to indicate what the storage molecules may be.

Mehler (218) and Gilmour (247) could detect the production of no long-lived substances when chloroplasts were illuminated briefly in the absence of oxidant. Mehler's results were negative but inconclusive. Gilmour, in more

extensive studies, corrected for the slow photoreduction of the fragments themselves, which he has previously discovered (232) and could then detect no long-lived storage of reducing power following illumination. He used single, short, high-energy flashes and then added ferricyanide in a mixing process which was complete in one second. His results demonstrated that less than one molecule of light-produced reducing agent remained per 2000 chlorophyll molecules. With oxidant present during the flash, considerable permanent reduction occurred. Thus, he could report no evidence for a long-lived intermediate formed without preliminary reduction. The energy storage intermediate leading to chemiluminescence, which appears to be produced without oxidation, was either undetectable or not present. His results do suggest, however, that the first oxidation and reduction reactions following uptake of light energy in the plant are closely coupled. Were this not the case, some slight amount of stored reducing power would be expected to appear as a result of the transient production of oxygen precursors during the flash. In a later series of studies, to be described in the next section, Gilmour and co-workers (247), obtained chemical evidence for long-lived intermediates which could be produced without preliminary oxidation by the Hill oxidant. The intermediates were produced only during flashes of high intensity and long duration and would not have appeared in the single flash experiments. These substances, if they actually exist, appear to be a possible source of the energy supporting chemiluminescence.

Effects of extrinsic factors.—One of the most powerful tools for studying the Hill reaction is that provided by chemical kinetics. In such studies, one systematically changes the conditions under which the system operates, and then attempts to develop a reaction mechanism which will quantitatively explain the observed variations in over-all velocity. This section briefly presents some of the recent studies of extrinsic factors on Hill reaction and photosynthesis velocity. Studies of some other extrinsic variables are reported in other sections of this review.

Horowitz (248) has recently studied the effect of deuterium oxide on the *Chlorella* Hill reaction with quinone as oxidant. He observed that the relative inhibitory effect of this inhibitor was the same at all light intensities in contrast to older observations that photosynthesis is inhibited by deuterium oxide only at high light intensities [see (40) for a review of this topic]. Horowitz' results indicate that deuterium oxide inhibits both the limiting light reaction and the limiting dark reaction of the Hill reaction. Since his parallel studies on *Chlorella* photosynthesis confirmed the older findings that only the limiting dark reaction was inhibited in photosynthesis, he concluded that the Hill reaction and photosynthesis must be different processes. In the course of his studies he made the interesting observation that 2,6-dichlorophenolindophenol worked as a Hill oxidant for *Chlorella* when the cells had undergone a preliminary freezing. The field is thus opened for the study of algal Hill reactions using oxidants other than benzoquinone.

Warburg & Lüttgens (249) observed that chloroplasts lost their photochemical activity after prolonged washing. This could be restored by the addition of low concentrations of chloride. Bromide was almost as effective, and iodide and nitrate showed some activity. Other anions and all cations tested were without effect. This so-called "chloride effect" has not as yet been explained to the satisfaction of all investigators. In the latest paper on the subject Gorham & Clendenning (250) proposed that chloride actually acts to stimulate the photochemical activity of chloroplasts rather than simply to prevent light inactivation [see Arnon & Whatley (251)]. Increased anion concentrations gave higher maximum rates together with a shift to a higher pH of the pH optimum. They pointed out the similarity of the chloride effect in the Hill reaction to chloride stimulation of starch hydrolysis by amylases and of salt respiration in root discs. Gorham and Clendenning noted that thiocyanate was inhibitory at low concentrations. This has been confirmed by Spikes *et al.* (252) in a series of studies of inhibitors of the Hill reaction. Univalent ions in general were found to inhibit by a simple mass-law mechanism. Di- and trivalent anions were either not inhibitory (at least up to concentrations where the chloroplasts were flocculated), or showed a weak inhibitory effect at lower concentrations and an activating effect at higher concentrations. Univalent cations also inhibited, but only at very high concentrations. Divalent cations were somewhat more inhibitory than the univalent cations.

Kosobutskaya & Krasnovskii (253) examined the effects of pyridine, dioxane, alcohols, phenol, and certain acids on the spectral properties and photochemical activity of isolated chloroplasts. Whittingham (254) showed that hydrocyanic acid rather than cyanide ions inhibits the carbon dioxide fixation mechanism in *Chlorella* photosynthesis. Ehrmantraut & Rabinowitch (207) examined the effects of a number of inhibitors on the Hill reaction with intact *Chlorella*. They made the interesting observation that the reaction was inhibited by malonate and that this inhibition could be reversed by fumarate.

Wessels (193), in an extensive paper, has considered a number of extrinsic factors in their effect on the Hill reaction of chloroplast fragments. Unfortunately, his investigations do not allow a clear distinction as to which limiting reaction is affected. He studied several inhibitors. In particular, he noted that the efficient inhibitory ability of orthophenanthroline is not directly related to its ability to act as a metal chelating agent. This confirms a similar conclusion reached by Spikes *et al.* (200). The latter authors report that orthophenantholine inhibits both light and dark limiting steps but with different efficiency. Fujimura & co-workers (255, 256) examined the effects of a number of variables including washing of chloroplasts [see also Wessels (193)], pH, temperature, length of storage, wavelength of light, age of plants. and oxidant conditions on the apparent redox potential of ground plants. Gerretsen (257) observed the dependence of the redox potential of macerates of *Avena* leaves on oxygen partial pressure and manganese ion concentration.

Just what the significance of such studies on these complicated systems is for photosynthesis is not clear.

Effects of flashing light.—The use of flashes of light rather than continuous illumination has proved to be a powerful technique in studying the mechanism of photosynthesis. This technique has recently been extended to the *Chlorella* Hill reaction by Clendenning & Ehrmantraut (258) and by Ehrmantraut & Rabinowitch (207). The maximum yield of the Hill reaction with saturating flashes was found on extrapolation to be the same as that for parallel photosynthesis studies. These data agreed with the classical studies of Emerson & Arnold (259). However, the relation between light intensity and yield was different for the Hill reaction and photosynthesis. The authors concluded from these studies and from quantum requirement measurements that the Hill reaction and photosynthesis were identical with respect to the rate-limiting enzymatic process. However, the large differences in yield with dark times shorter than those giving maximum yield could very well be interpreted as indicating differences between the two reactions. Also, the anomalous effects of quinone (as previously discussed) in the concentration ranges used are difficult to evaluate in flashing light studies.

Emerson & Arnold (259), who worked with very short flashes (10^{-5} sec.), observed a temperature dependent "working time" for the dark reaction of 0.02 sec. The maximum yield per flash was independent of temperature, however, and amounted to one molecule of carbon dioxide fixed per 2480 molecules of chlorophyll, This gave rise to the idea that the energy absorbed by this number of chlorophyll molecules contributed to some center at which the carbon dioxide molecule was reduced. Gaffron & Wohl (260), on the basis of these results, proposed that there existed a "photosynthetic unit" of about a thousand chlorophyll molecules and one enzyme molecule which formed the natural unit mechanism for fixing carbon dioxide. This model is no longer tenable as a unit for carbon dioxide fixation. However, it is still of fundamental importance as a model for the geometrical and morphological unit involved in the collection of light energy either as electronic quanta or as chemical intermediates. A number of the subsequent studies supported the findings of Emerson and Arnold with respect to the dark times required for maximum yields. Some workers, however, reported that longer dark times were required to obtain maximum yields [see, for example, Briggs (261); Kennedy (262)]. In 1949 Tamiya & Chiba (263) published a very extensive study of *Chlorella* photosynthesis in flashing light in which they could not duplicate the results of Emerson & Arnold (259) except under special conditions at nonsaturating light intensities. They used a sector wheel for obtaining intermittent light which produced longer flashes than those of the discharge tube used by Emerson and Arnold. Under these conditions they found that dark times much longer than 0.02 sec. were required for maximum yield. The maximum yield per flash was also found to be temperature dependent. Tamiya suggested that the earlier work was carried out with

light flashes which were too weak to saturate the dark enzyme systems. In turn, it was suggested by others that Tamiya's experiments permitted operation of the dark enzyme during the long light flash. The participation of induction phenomena was also suggested to account for these different results (207). There can be no doubt, however, that Tamiya's results must be taken seriously. Phillips & Myers (226) in studies on the efficiency of light utilization in *Chlorella* growth concluded that the picture presented by Emerson & Arnold (259) is oversimplified. They obtained increased utilization of light energy by extending the dark periods to 67 msec. and greater (226). Gilmour, Lumry & Spikes (247, 264, 265), in flashing light studies on the Hill reaction with chloroplast fragments of sugar beet, obtained both types of results as described above. At low flash intensity or short flash time, or both, the working time and maximum yield per flash were similar to those in the work of Emerson & Arnold (259). Flashes of high intensity and of long duration gave results qualitatively similar to those of Tamiya & Chiba (263). Gilmour *et al.*, on the basis of a kinetic treatment of their data suggested that the latter flash conditions lead to the production of new intermediates in the reaction sequence prior to the step involving reduction of the added oxidant. One intermediate, which may be an extraneous energy storage material, persisted for many msec. Another intermediate decayed by a temperature independent process which indicated that it was a high energy substance. It may be remarked that these intermediates are produced under conditions which makes their existence in the steady-state Hill reaction highly probable. We can suppose that a photoproduct produced at the start of a light flash can be "processed" by the dark reaction and then return to receive a second quantum of light energy. If we further suppose that it must return to the same site for the second photoreaction, it would have a very low probability of finding a second quantum except at very high light intensities or at long flash time. In this way the need for long intense flashes might be explained. The effects of reaction conditions including the presence of deuterium oxide and inhibitors were examined in this work. The results also suggest the possibility that there are two different sites for oxidant reduction [see Franck (266)].

Allen (267) has recently studied the Hill reaction and photosynthesis of *Scenedesmus* under highly anaerobic conditions (10^{-6} mm. Hg of oxygen). He used the phosphorescence-quenching technique to follow oxygen evolution. It was found that a single 0.5 msec. flash would lead to the evolution of oxygen in the Hill reaction but not in photosynthesis. A 25 msec. flash with the same total energy as the short flash resulted in oxygen evolution in both systems. Two short flashes spaced as much as 1 sec. apart increased the yield in the Hill reaction 30 per cent over the yield obtained with the two flashes spaced several minutes apart. The yield per short flash in the Hill reaction with 5×10^{-4} M quinone as oxidant was one molecule of oxygen per 3×10^4 chlorophyll molecules. This yield was constant over a wide range of light

intensities. A single short flash resulted in oxygen evolution in photosynthesis in the presence of low intensity continuous illumination. The yield, however, decreased with flash intensity. The yield per short flash in photosynthesis under these conditions was approximately one oxygen molecule per 5×10^4 chlorophyll molecules. Illumination during photosynthesis with a long and a short flash showed the same increase over the sum of yields for isolated flashes as was shown by the Hill reaction. In both reactions separated flashes produced more oxygen than simultaneous flashes. The short flash yield was reduced 50 per cent when it followed the long flash by 20 sec. The effect was not due to a deficiency of oxygen in the reaction mixtures as was shown by adding oxygen. These results indicate that very long-lived intermediates are produced in the first flash which can increase the efficiency of the subsequent flashes. The Hill reaction does not require previous "priming" by a long flash, but photosynthesis cannot proceed in short flashes unless priming has occurred. Allen has interpreted his results in terms of Franck's narcotization hypothesis together with some additional considerations based on flash shape. However, the similarities existing between his work and that of Gilmour *et al.* (247, 264, 265) suggest that there may be an alternate explanation. The conditions of total illumination required to support photosynthesis in Allen's work are very similar to those required for Gilmour's intermediates. It seems entirely possible, therefore, that the latter intermediates are essential components of photosynthesis. The Hill reaction can proceed without them, but photosynthesis apparently cannot. Furthermore, Gilmour (247) was unable to detect stored reducing power after illumination in the absence of Hill oxidant. His technique was sufficiently sensitive to detect any of the long-lived intermediates noted in his later work and in Allen's work. Thus, it appears that a preliminary reduction is necessary to stabilize these intermediates, a preliminary reduction which both photosynthesis oxidants and Hill reaction oxidants can support. The photosynthetic process, however, cannot proceed to completion unless the second reduction following the production of intermediates can occur. These observations suggest again that two reduction processes are required in photosynthesis. Both of these must be coupled to the oxygen producing system in such a way that neither carbon dioxide reduction nor oxygen production can occur unless both processes take place. Such a mechanism would require cyclic systems for both part-processes. The Hill reaction, on the other hand, appears to go to completion after reduction at only the first site. It is also possible that the chemiluminescence described by Strehler & Arnold (245, 246) is due to the long-lived intermediates, perhaps the 20 sec., half-life material suggested by Allen's results (267). The flashing light studies of Ehrmantraut & Rabinowitch (258) and the well-known fact that the Hill reaction has no induction period give clear evidence that photosynthesis and the Hill reaction are not identical processes, even with allowance for the carbon dioxide fixing system in photosynthesis. Allen's experiments also

bear on the mechanism of the induction period and its dependence on oxygen. His photosynthesis measurements were carried out at such low oxygen pressures that a long induction period would be expected. Nevertheless, the short and long flash together were able to produce oxygen in a good yield.

It should be emphasized here that in comparisons of photosynthesis and the Hill reaction, especially when chloroplast fragments are used for the latter, every attempt should be made to operate at a pH less than 7. The Hill reaction shows relatively simple behavior under these conditions. At higher pH values the reaction is difficult to control and the rates depend on the particular batch of leaves from which the fragments are prepared as well as on factors as yet not fully understood. Burk *et al.* (268, 269) reported a solarization (light inhibition) with *Chlorella* at high light intensities which limits the yield in both flash and steady illumination. In an earlier note he criticized previous studies of the maximum yields in flashing light as being too small because of the use of too low flash intensities (269). The recent note attributes the low maximum yields to too high intensities which produce solarization. Steemann Nielsen (270) continued his earlier studies of light inactivation phenomena which are perhaps the same as those described above. He attributes the effect to a photooxidation process which inactivates both enzymatic and photochemical components of photosynthesis. The effect is especially pronounced when light intensity is reduced from some high initial value. Solarization thus appears to involve the effects of oxygen. The phenomenon is not well understood and should be investigated thoroughly. The precise comparison of Steemann Nielsen's results with those of other workers is difficult since he used the Winkler method for measuring oxygen and consequently had to employ long experimental periods.

The photosynthetic unit of chlorophyll molecules remains an attractive concept. It may contain not 30,000 or even 2500 chlorophyll molecules, but perhaps only a few hundred. Duysens has recently applied Förster's sensitized fluorescence theory (see first section) to his data. Although the calculations are only approximate, they indicate a unit of 200 chlorophyll molecules (168). Thomas *et al.* (271) studied the Hill reaction velocities of approximately monodisperse chloroplast fragments of decreasing size. Under both high and low light intensity the reaction rate decreased with particle size only below a critical size of particle which contained approximately 100 chlorophyll molecules. Burk *et al.* (269), on the other hand, interpret the photosynthetic unit as consisting of a single chlorophyll molecule.

Quantum Requirements

Probably no problem in modern plant physiology has resulted in more controversy than the matter of the minimal quantum requirement for photosynthesis. The first measurements were made over thirty years ago, and as experimental techniques and theoretical arguments have improved the cleavage between the proponents of a high quantum requirement and a low

quantum requirement has increased. At the present there seems to be no way of reconciling the results of the two groups. The subject has reached the state of a special science in itself, and the present authors are certainly not competent to evaluate properly much of the work in the field. However, because of its importance to other aspects of photosynthesis, it is necessary to discuss briefly the most recent work on quantum requirements.

Quantum requirements for the Hill reaction.—As yet there is little controversy concerning the quantum requirement for the Hill reaction since no one has found either a four quantum requirement or the one quantum phenomenon of Warburg and co-workers (as discussed in the next section). Ehrmantraut & Rabinowitch (207) observed requirements for the *Chlorella* Hill reaction which averaged around 10 quanta per oxygen molecule in the best determinations. The requirement for isolated chloroplasts from *Phytolacca americana* average 10.6 quanta per molecule of oxygen. The nature of the oxidant (quinone, ferricyanide, Hill's solution) had little effect. It was pointed out in the section on the mode of oxidant participation that quinone strongly inhibits the limiting photochemical reaction at higher concentrations. Thus, the oxidant concentration could influence the quantum requirement. No attempt was made in the above work to determine the dependence of the quantum requirement on light intensity. The chloroplast Hill reaction rate attains saturation at relatively low light intensities. For this reason the minimum quantum requirement could be attained only by extrapolating rate data to zero light intensity.

Wayrynen (272) studied the quantum requirement of the Hill reaction of sugar beet chloroplasts. Ferricyanide at low concentration was used as the oxidant. Rates were measured potentiometrically (210) with light at 675 mμ. A requirement of 8.3 quanta was found by extrapolation of the data as described above. This value was independent of temperatue but was dependent on pH. Optimum results were obtained at pH 6.3. Further improvements in the preparation of chloroplasts and in the control of reaction conditions may well lead to even lower requirements.

Wayrynen observed that the quantum requirement for the chloroplast Hill reaction rose to a maximum at 575 mμ and decreased again at 475 mμ the shortest wavelength used. There was no indication of a rapid drop in quantum requirement at shorter wavelengths as Warburg found using spinach chloroplast preparations and with quinone as oxidant (273). He reported a change in quantum requirement from 70 at 400 mμ to 100 at 644 mμ and offered this as evidence for a fundamental difference between photosynthesis and the Hill reaction. Differences between the quantum requirements of photosynthesis and the Hill reaction may, of course, exist. It should be noted, however, that Warburg's values (which are by far the highest reported to date) were obtained at high quinone concentrations (.0053 M) where secondary inhibitory effects are observed. Chen (274) made careful determinations of the action spectrum for the Hill reaction of chard chloroplasts and

found a large proportionate difference between absorption and the action spectrum in the 575 mμ region where Wayrynen observed the highest quantum requirement No explanation for the energy loss in this region has been established

Quantum requirements for photosynthesis.—Several new papers on this important subject have appeared since the excellent review last year by Brown & Frenkel (8). Warburg and co-workers have recently presented what, to us, appears to be the best experimental support thus far for the four quantum requirement (275). They worked with rapidly growing cultures of *Chlorella* in their usual medium at pH 4.2 at high light intensity. Under these conditions the photosynthetic rate was very high so that problems concerning light-produced changes in respiration could be ignored. The algae reached light saturation only at unusually high light intensities. This may be related to the finding of Tamiya *et al.* (276), that *Chlorella* exhibits two growth phases, one of which contains more chlorophyll and demonstrates greater photosynthetic rates than the other. Warburg's recent paper described several other technical innovations such as the use of argon rather than nitrogen, but the results still indicated an overall quantum requirement of about four. Five per cent carbon dioxide was employed, and some of the experiment ran for several hours. Quantum requirements less than four were occasionally observed and in another paper, which discusses experiments carried out with a similar technique, an average of 3.88 was reported. Warburg concludes that the best value is probably less than three (277). There appears to be some question concerning the effects produced by carbon dioxide at these high concentrations [Franck (266)]. Steemann Nielsen (270, 278) has reported data which can be interpreted as indicating a block of respiration by high carbon dioxide concentration in light which he says makes the requirements published by the Warburg group threefold too small. Warburg's work as cited above, however, describes experiments in which respiration corrections, no matter what they may be, should still be negligible. Briggs & Whittingham (279, 280) on the other hand found that high carbon dioxide concentrations during photosynthesis had no effect on the photosynthetic rate. However, they did find an inhibitory effect of high carbon dioxide concentration in the growth phase. Four per cent carbon dioxide resulted in a low photosynthetic rate which, after several hours, increased to a four or fivefold greater rate. They interpreted their results in terms of an inhibitor which is removed photochemically, but only at low carbon dioxide concentrations. Franck has also recently discussed the uncertainties which may result in quantum requirement studies carried out at low carbon dioxide concentrations (266).

Recent studies indicate that the concentration of carbon dioxide required for maximum photosynthesis rates may be higher than was generally believed. Both Österlind (281) and Rosenberg (282, 283), although they used different methods, arrived at this conclusion. Rosenberg used a glass elec-

trode technique to follow carbon dioxide changes by means of very thorough determinations of the relation between carbon dioxide concentration and pH. He found that photosynthesis rates fell off rapidly at saturating velocity due to pH changes and to a decrease in the amount of carbon dioxide available. Maximum rates occurred in the pH range 6.6 to 7.0. However they varied with light intensity, carbon dioxide concentration, cell concentration, and bicarbonate concentration. Warburg (284) had previously observed that the low yields in 0.2 per cent carbon dioxide could be raised to a maximum at 2 per cent carbon dioxide.

The Warburg-Burk group continues to publish data which substantiate their "one-quantum" mechanism in which three oxygen molecules are produced and then reused for each one permanently released. One quantum is supposed to provide enough energy to produce each of the three oxygen molecules which are reused. In one of their latest papers [Damaschke et al. (285)] they used a technique derived from polarography to measure changes in oxygen concentration. High speed stirring was used to give what is known as "convective" polarography (286) in which the normal polarographic slow step (diffusion) is replaced by some other step which is not especially dependent on the nature of the material being reduced in a given potential range. It should be pointed out that problems might arise with this technique resulting from photoeffects at the electrodes. This procedure should reduce the time lag in the response to changes in oxygen concentration to a very low value which would eliminate arguments based on the low speed of response of manometers [see Kok et al. (287)]. The new technique as used by the Warburg—Burk group gave results which confirmed their earlier studies since quantum requirements of approximately 3 to 4 were found.

The best experimental evidence against the "one quantum" hypothesis still seeems to be the conventional oxygen polarographic studies of Brackett and co-workers (288, 289) and the studies of Brown (290) in which he was unable to detect any effect of illumination on the uptake of labelled oxygen from the gas phase by algae. Brackett et al. carried out experiments very similar to those of the Warburg-Burk group as described above. Their technique permitted a 10 sec. resolving time and thus were able to study transient phenomena in some detail. The results, which were quite different from those described by Burk et al. were interpreted to mean that respiration changed as a result of illumination. The change was delayed, however, so that periodic illumination could be accompanied by respiration changes completely out of phase with the illumination pattern. As a result, the average value of respiration under certain conditions could be higher in the dark than in the light. The quantum requirement was independent of light intensity and values as low as 6.5 were obtained. The lowest values were not considered to be significantly different from the average requirement of about eight. However, requirements of approximately four could be calculated by using an alternative procedure. The arguments leading to the elimination

of the lower quantum requirements found in this work are not completely indisputable, so that the results do not represent a wholly satisfactory argument against the "one quantum" theory. Brown (290) used the mass spectrometer technique in a region of oxygen partial pressure at which its accuracy and resolving time were not optimal. The arguments based on Brown's data do not rest on accuracy or time resolution but rather on the average amount of tracer oxygen incorporated during a number of periods. His general failure to find a net stimulation of respiration by light is, of course, a valid argument against the one quantum hypothesis. Brown & Webster (291) reported a 150 per cent stimulation of the respiration of the blue-green alga *Anabaena* by high light intensities when the oxygen partial pressure was sufficiently high. A photoinhibition of respiration occurred at oxygen concentrations below 0.5 per cent. This is the only organism studied by Brown which showed a pronounced photostimulation of respiration.

It is conceivable, if the "one quantum" idea is correct, that the efficiency of photosynthesis would depend on the partial pressure of oxygen. The Warburg-Burk group found no such dependence even down to 0.3 per cent oxygen. Effects which did appear at lower concentrations could be attributed to induction phenomena. The induction phases of photosynthesis (which do not occur in the Hill reaction) increase with the length of storage time under anaerobic conditions. As is well known, Gaffron, Franck, and co-workers attribute such induction periods to a narcotization produced by fermentation products. Recently Hill & Whittingham (292) studied the induction period with a technique which used hemoglobin to follow oxygen changes. They detected a short induction period (half-time 30 sec.) following aerobic incubation in the dark, and a longer period (half-time about 2 min.) following anaerobic storage in the dark. These induction phases developed to full value in only a few minutes of incubation. In the interpretation of their results the authors concluded that oxygen (and carbon dioxide) is necessary for photosynthesis. They set a lower limit of 2 mm. Hg for the minimum oxygen pressure necessary for photosynthesis. These results do not agree with the recent findings of Allen (267) who showed that, even with oxygen partial pressures as low as 10^{-6} mm. Hg, oxygen was produced by a single short flash of light following a single flash of longer duration (see previous section). With continuous illumination and a constant oxygen pressure of 4×10^{-3} mm. Hg he was able to demonstrate photosynthetic rates at least as great, and sometimes greater, than those found with the same cultures (*Scenedesmus* and *Chlorella*) in air. These results provide the most convincing evidence to date that oxygen is not required for photosynthesis and confirm the results of older experiments of the same type. The ability of oxygen to reduce or eliminate induction periods appears to be an artifact resulting from the substitution of oxygen for substances produced by the plant in the preliminary stages of photosynthesis. Allen discussed his results in terms of narcotization theory and noted the possibility that 6,8-dithioloctanoic

acid (reduced thioctic acid) may need to be oxidized before oxygen can be liberated. This last proposal is based on the interesting suggestion by Franck that thioctic acid may act as a coenzyme for the oxygen liberating catalyst (his catalyst C) rather than as the primary energy pickup substance.

The evidence presented in Warburg's latest paper (275) is very impressive, although possibilities for error are undoubtedly still present. For example, high carbon dioxide concentrations were employed. In considering the acceptance of Warburg's values for the quantum requirements of photosynthesis, we must first ask what we are accepting. Certainly, no serious consideration should be given to claims of 2.7 or 2.8 quanta per oxygen molecule. Such values would require perfect efficiency in the utilization of light energy, a "thermodynamic perfection of nature" as Burk describes it (293). Even if photosynthesis were only a two step process proceeding at a very slow speed, it is doubtful that there would be much point in considering a quantum requirement of 2.7. With the larger number of steps which must exist in the real process, this low limit can certainly be raised to 3 quanta, experimental observations notwithstanding. Quite another question arises if we are asked to consider a four quantum requirement. In a recent elaboration of some of his earlier ideas on this question, Franck (266) has calculated that a minimum of 67 kcal. of heat energy must unavoidably be produced during a four quantum photosynthesis process. The total free energy requirement then would be 117 kcal. plus 67 kcal. plus any entropy loss which might occur. This sum is too large to be balanced by the 164 kcal. of free energy available in four photons of the longest wavelength of light which will support the photosynthesis of green plants with the same yield as light of shorter wavelengths. We will discuss this paper in some detail since it appears to provide one of the best available arguments against the very low quantum requirement proposed by the Warburg group.

In first suggesting this thermodynamic type of argument, Wohl calculated a minimum requirement of 180 kcal., although he used a different theoretical mechanism (294). The thermodynamic entropy loss is probably quite small in photosynthesis, although it should be remembered that part of the initially available free energy will be lost simply because the real process proceeds at a rapid rather than at an infinitely slow rate. The energy discrepancy of 67 kcal. per oxygen molecule as suggested by Franck depends to a certain extent on the particular theoretical mechanism for photosynthesis chosen. The reasonable basis of Franck's arguments which indicate that four quanta cannot be sufficient must be admitted. The initial loss of energy is attributed to the unavoidable production of vibrational excitation at the same time that electronic excitation takes place. The part of the light quantum which is converted to vibrational energy of the excited singlet state exceeds the amount of vibrational energy which the excited molecule would posses as a result of thermal collisions. That is, it is hotter than its neighbors and therefore must cool rapidly to the average surrounding temperature.

The excess of vibrational energy is estimated as 3 kcal. by Franck which gives a net loss of 12 kcal. in a four quantum process.

A second source of loss is related to the fact that photosynthesis is very efficient. The single steps involved must go forward considerably more easily than backwards. Three mechanisms will permit this "ratchet" type of action: (a) a large free energy barrier facing the returning product molecules; (b) a rapid irreversible reaction of the products; and (c) a dilution of the products if the reaction produces at least two partners. If the second step of the light process involves crossing to a metastable excited state, Franck believes that mechanism (a), at least, plays a role in limiting the back reaction, and he suggests that the metastable state lies 3 kcal. lower than the singlet excited state. This gives a possible loss of 12 kcal. If the reaction does not proceed through the known metastable state but through some as yet unknown electronic state induced by a pickup substance, mechanisms (b) or (c) might conceivably limit the reversibility so that the intermediate states could lie at the same energy level as the singlet excited state. Since the subsequent dilution or subsequent irreversible reaction would also result in a loss of free energy, it is difficult to visualize how any large reduction in the energy loss could be secured with the alternate mechanisms.

Franck next suggests that 16 kcal. will be the minimum loss produced in the four chemical reactions of the four molecules of primary energy conversion substances. Assuming that these reactions occur from one energy state (excited electronic state) which contains at least as much free energy as any transition intermediate in the course of these reactions, an irreversibility in the case of mechanism (a) could only be achieved by a net loss in free energy. This would result from the fact that the free energy of the products must lie at a lower level than the free energy of the original excited state. This follows from the requirement for a free energy barrier to restrict the back reaction which, in this case, can only consist of the free energy necessary to get back to the level of the original excited state. If the levels of reactants and products are equal, nothing hinders the back reaction in the event that the products remain closely positioned in space and no rapid reaction of products occurs. We may envision an alternate way to provide the necessary barrier for the return reaction by imposing a barrier for the forward reaction of the electronic state. Such a mechanism is uncommon, but not necessarily impossible. Whether or not it can be shown to be inconsistent with the facts of photosynthesis is another matter. Mechanism (c), however, appears as a better alternative. The products might be slightly displaced from each other in space and held apart in a new state of very nearly the same energy as the reactant state. Alternatively one or both could be carried away in subsequent reactions as, for example, the loss of a hydrogen atom to an oxidizing participant of the overall process. The free energy loss in such processes might be as low as 2 kcal., but it is probably closer to 3 kcal. This results in a small saving of 4 kcal. compared to Franck's estimate.

The heat loss for oxygen production based on the dismutation of photo-peroxides is estimated as at least 20 kcal. by Franck. If, however, one can temporarily ignore existing interpretations and theories of photosynthesis to ask just what is the minimum conceivable energy loss in the reaction sequence which produces oxygen, then 20 kcal. does not appear to be the lowest possible estimate. Consider, for example, the hypothetical sequence of events shown in Illustration E. There is no reason to suppose that this mechanism has anything to do with photosynthesis, but it could conceivably be modified by the introduction of allowable chemical properties and alternate reactants to explain most of the observations that can be explained by other theories. Reaction conditions and energy level balances would have to be remarkably well adjusted to allow the evolution of oxygen without any large free energy changes between the several steps. Nevertheless, we cannot exclude the possibility that this would happen. The use of a cyclic peroxide is just one of many possible mechanisms. This argument was presented to
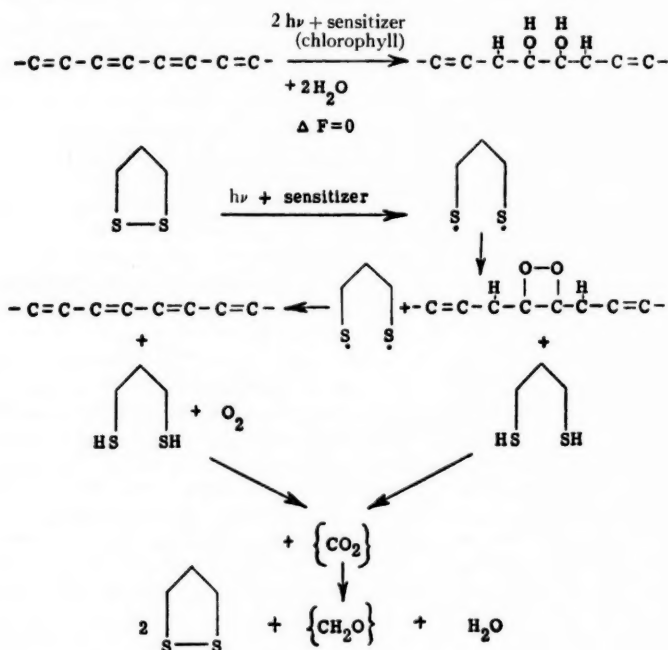
Illustration E

show that Franck's estimate of 20 kcal. for the energy loss of oxygen evolution may be considerably too high. A loss of 5 kcal. would perhaps be a more reasonable absolute lower limit. Franck proposes a 6 kcal. loss in the dismutation of radicals during carbon dioxide reduction, which appears reasonable. Nor can we reduce the 13 kcal. loss which Franck has estimated for the dark reactions of the carbon dioxide side of photosynthesis. These reactions are generally better understood than those treated above, so that there is less room for disagreement.

Our reestimation of the individual energy losses in photosynthesis as detailed above would, at most, decrease the total energy loss from 67 to 45 kcal. This would give an overall energy requirement of 162 kcal. If it is assumed that the entire quantum of energy can be delivered to the chemical systems, and that the system operates with maximum (thermodynamic) efficiency, there would be just enough energy on this basis to supply the requirement. What energy is lost could not be trapped for reutilization, and the extreme conditions assumed make a quantum requirement smaller than four for photosynthesis implausible if not impossible. We conclude that anything less than four quanta could not support oxygen production without additional energetic assistance, and thus on a long-term basis could not support photosynthesis. We agree with Franck that the resaonable quantum requirement for photosynthesis based on his estimate and our reestimate is at least five or six.

In the same paper Franck (266) continues the discussion of energy utilization to emphasize the probabliity that more than one quantum must be utilized for each hydrogen atom of the phosphoglyceric acid reduction process. The bulk of his paper is devoted to a detailed attempt to reconcile the various experimental determinations of quantum requirements. We will not attempt any further critical discussion of this paper which merits study both by specialists and nonspecialists. The central idea is that respiration produces phosphoglyceric acid and related compounds at the expense of the stored metabolic energy of the organism. When these materials are present in high concentrations relative to the regular carbon dioxide acceptor molecules of photosynthesis, they are preferentially reduced. [Phosphoglyceric acid or diphosphoglyceric acid can be reduced with a smaller expenditure of light energy in a manner which does not change the mechanism of oxygen production.] In this way the apparent quantum requirement could be decreased by as much as one-half. An extensive discussion of the possible errors in the experiments of the Warburg group, both demonstrated and postulated, is given together with a correlation of their observations with Franck's idea of "short-cut" photosynthesis. Unfortunately, Warburg's most recent paper (277) appeared after Franck's paper and thus was not discussed. Some of Warburg's experiments continued for three hours or more at very high rates of photosynthesis without deviating from a quantum requirement of four. If Franck's latest hypothesis is correct, Warburg's *Chlorella* must

have been provided with astonishingly large reservoirs of carbohydrate. It is not entirely clear just how the Hill reaction fits into Franck's picture. Presumably, Hill oxidants would substitute for phosphoglyceric acid and might, therefore, be expected to permit the lower quantum requirement. So far, the requirement does not appear to be lower than eight. It is quite possible that the total energy requirement for the Hill reaction is just as great as that for photosynthesis. More energy is lost in the reduction process with oxidants thus far employed than in photosynthesis, but the total difference in energy for the two processes appears, from what has been said in the previous section, to be no more than about 12 kcal.

Additional mechanisms for photosynthesis have been proposed by R. Schenck (295), Levitt (296), and G. Schenck (59).

Most experimental attempts to reconcile the divergent results of studies of quantum yield, oxygen, and carbon dioxide effects in photosynthesis have been directed toward improving the experimental procedures for measuring the velocity of photosynthesis. Comparatively little attention has been directed toward control of algal growth. Since the experimental techniques used to support both sides of the three disputes have become very good, the attention of the nonspecialist is attracted to the possible importance of growth variables, many of which do not appear to have been studied. One wonders if any of the aggravating questions can be settled without an extensive investigation of the many factors which must be controlled in order to provide an internationally reproducible sample of algae.

<center>MISCELLANEOUS</center>

Intermediate process in carbon dioxide fixation.—The chemical pathway of assimilated carbon in plants was thoroughly covered in the excellent review of Brown & Frenkel (8). The mechanism of carbon dioxide fixation in both plants and animals was recently reviewed by Ochoa (297), and Maurandi reviewed the application of radioautographic and chromatographic methods to studies on photosynthesis (298). Bassham, Benson & Calvin (299) presented a brief review of the techniques used in studying carbon dioxide fixation in algae with isotopic carbon.

Benson *et al.* (300) examined the kinetics of the formation of intermediate compounds in photosynthesis under steady state conditions, while Lynch & Calvin (301) studied the fixation of carbon dioxide by *Euglena*. The action of several antibiotics and other biologically active substances on the carbon dioxide fixation of *Scenedesmus* was investigated by Havinga *et al.* (302). Zweifler (304) determined the distribution of radioactivity in photosynthetically-produced ribulose, while Abraham *et al.* (309) examined the distribution of activity in photosynthetically-prepared glucose labeled with C^{14} (309). Gibbs (308) made a critical examination of the distribution of C^{14} in sucrose, alanine, and malic acid from sunflower plants photosynthesizing

at high and low light intensity. Additional compounds in green plants of possible significance in photosynthesis were identified including nucleotide coenzymes [Buchanan *et al.* (303)] and sucrose phosphate [Buchanan (305)]. Vernon & Aronoff (306) studied the translocation of labelled compounds produced in photosynthesis in the soybean plant. Racusen & Aronoff (307) examined the dark reactions involving labelled compounds in soybean leaves after a period of photosynthesis. Doman (310) studied the intermediate products of photosynthesis in the bean plant using C^{14} and claimed that the primary product of photosynthesis could not be a substance of low molecular weight. Doman *et al.* (311) examined the intermediates in photosynthesis with labelled carbon dioxide in 12 families of plants. Voskresenskaya (312) reported that the relative concentrations of different compounds formed during photosynthesis was altered by the spectral quality of the light used.

Isotope effects in carbon dioxide fixation.—Reaction rates obtained in the same reaction with different isotopes of the same element are often quite different. Obviously then, if isotopic discrimination of a high order existed for carbon isotopes in the photosynthetic fixation of carbon dioxide the results of tracer experiments (especially kinetic experiments) would be difficult to interpret. The extent of isotopic discrimination in single-step reactions is usually quite small with atoms as large as those of carbon. Photosynthesis, however, apparently involves a considerable series of reactions, so that isotopic fractionation could become cumulative and large. An excellent review on carbon isotope effects in biological systems, including photosynthesis, has appeared recently [Buchanan *et al.* (313)]. Van Norman & Brown (314) used a recording mass spectrometer to make almost continuous measurements on the uptake of carbon dioxide containing different isotopic forms of carbon by *Chlorella* suspensions and by barley leaves. An isotopic discrimination was found in which $C^{12}O_2$, $C^{13}O_2$ and $C^{14}O_2$ were apparently metabolized at the relative rates of $1.00:0.96:0.85$, respectively. The discrimination for $C^{14}O_2$ is thus several times greater than would be expected for a simple reaction. Baertschi (315, 316) examined the fractionation of naturally occurring carbon isotopes in green plants. Data on the C^{12}/C^{13} ratios of some 105 different species of naturally growing plants show that the carbon isotopic fractionation is a function of the habitat (tropical rain forest, stagnant water, marine, nonstagnant water, and desert) rather than the systematic position of the plant [Wickman (317, 318)].

Carbon dioxide fixation with cell-free preparations.—It has been shown that illuminated chloroplasts can reduce di- and triphosphopyridine nucleotides. If supplemented with the proper enzyme systems the chloroplast-diphosphopyridine nucleotide systems (see section on Hill reaction) can bring about the photochemical reduction of pyruvate to lactate, oxalacetate to malate, 1,3-diphosphoglycerate to hexose diphosphate and, in addition, the reductive amination of α-ketoglutarate. If the chloroplast-triphosphopyridine nucleo-

tide system is supplemented with the proper enzymes it can cause the photo-chemical formation of malate and succinate from carbon dioxide and pyru-vate and the reductive carboxylation of α-ketoglutarate to citrate [Vishniac & Ochoa (212, 213)]. A TPN-linked photochemical incorporation of carbon dioxide into 1-malate in a system containing washed chloroplasts, green leaf "malic" enzyme and pyruvate has been shown using $C^{14}O_2$ and paper chromatography of the reaction products. Oxygen gas was evolved by the system [Arnon & Heimbürger (215)]. The above results do not, of course, prove that photosynthesis proceeds through any of the reactions described.

Cell-free preparations of leaves show an increased rate of carbon dioxide fixation upon illumination. Most of the carbon dioxide is incorporated into phosphoglycerate and phosphopyruvate [Fager (318a)]. The rate, however, is very small compared to the photosynthetic rate of the same material. Oddly enough, these preparations were unable to carry out the Hill reaction. Cytoplasmic proteins seemed to be essential for the fixation reaction (318b). It has been reported that isolated chloroplasts reduced carbon dioxide by a series of reactions which proceed via carboxylic acids which do not reduce Cu, then uronic acids, then ketoses [Vinogradov *et al.* (216); also see Gerret-sen (257); and Boichenko & Baranov (319)].

Tolbert & Zill (320), in an important contribution have recently shown that cell-free protoplasm from *Chara* and *Nitella* fixed radioactive carbon dioxide at 12 to 15 per cent of the rate of the whole cell. Cut cells (both ends cut off and vacuolar sap carefully removed) fixed radioactive carbon dioxide at rates comparable to those of intact cells. In both cases the carbon dioxide was reduced in the light to sucrose and hexose phosphates. The two systems were, in general, similar except that the cell-free protoplasm did not form labelled pentoses or sedoheptuloses as did the cut cells and whole cells. The authors suggested that the system most sensitive to disruption of the cell was that involved in the regeneration of the "C-2 acceptor" for carbon dioxide fixation.

Algae metabolism and photosynthesis.—Algae, such as *Chlorella*, have been extensively used for studies in photosynthesis. As is well known, the photo-synthetic characteristics of a plant depend somewhat on the culture condi-tions employed. Thus, any information on the growth and metabolism of algae may be of importance in interpreting their photosynthetic behavior. This section consists of a brief listing of recent papers in this field. Walker (321) examined the inorganic micronutrient requirements (calcium, stron-tium, copper, molybdenum) for *Chlorella*. Finkle & Appleman (323) showed that magnesium deficiency arrested cell division without interfering with protoplasm synthesis, thus producing algae cells with up to twenty times the usual volumes. The assimilation of ammonia and organic nitrogen com-pounds by *Chlorella* was investigated by a number of workers [Syrett (324, 325); Schuler *et al.* (326); and Arnow *et al.* (322)]. Sorokin & Myers (327)

isolated a high-temperature *Chlorella* strain which showed optimum growth at 39°C. rather than at the usual 25°C. The isotope effect on the incorporation of tritium oxide into growing *Chlorella* was found to be much smaller than would be expected [Weinberger & Porter (328)]. Van Niel *et al.* (329), in studies on the photochemical reduction of nitrate by *Chlorella* concluded that the nitrate acts directly as an alternate hydrogen acceptor in photosynthesis [see also Davis (330); and Evans & Nason (217)]. New compounds isolated from *Chlorella* include ergosterol [Klosty & Bergmann (331)] and arginosuccinic acid [Walker (332)]. *Chlorella* contains arginosuccinase which catalyzes the formation of arginosuccinic acid from arginine and fumarate [Walker & Myers (333)], as well as transaminase systems [Millbank (334)] and the usual enzymes for carbohydrate metabolism [Holzer & Holzer (335)]. Eny (336) examined the effects of a number of substrates, inhibitors, etc., on *Chlorella* respiration. Brown (290), using isotopic oxygen showed that light had no marked effect on the respiratory rate of *Chlorella* (as well as other algae and vascular plants). Kok (337) made detailed studies on the efficiency of conversion of light into organic matter by *Chlorella* over extended periods (24 to 180 hr.). Optimum efficiencies were 20 to 24 per cent for sodium light. Whittingham (280) examined the effect of carbon dioxide concentration on the rate of photosynthesis of *Chlorella*. Briggs & Whittingham (279) examined the photosynthetic rate of *Chlorella* at high light intensities and low carbon dioxide concentrations.

Tamiya *et al.* (276) made very careful observations on the course of growth of *Chlorella* cultures and found that the cells assumed two distinct forms with different metabolic aad photosynthetic properties. A spectroscopic method was used to examine the induction phenomenon in *Chlorella* by Hill & Whittingham (292). Spruit (338) carried out an extensive and detailed study of the interactions of light, carbon dioxide, and oxidation-reduction potential with respect to *Chlorella* photosynthesis. Davis (339) obtained biochemical *Chlorella* mutants with ultraviolet radiation which required reduced carbon for growth even though they appeared to have normal chlorophyll.

A number of papers have appeared recently on algae other than *Chlorella*. Arnon & Wessel (340) examined the inorganic nutrition of *Scenedesmus* and made the unexpected observation that vanadium was an essential element for this organism. Arnon *et al.* showed that molybdenum was also essential (341). A number of diatoms were found to be able to grow heterotrophically (342). The assimilation of acetate by the blue-green alga *Nostoc* was studied by Allison and co-workers (343). Pure cultures of the green alga *Ankistrodesmus* were found to reduce nitrate to nitrite (344). The oxidative metabolism of the flagellate *Euglena* was studied by Danforth (345), while the respiration of the blue-green algae *Anabaena* was investigated [Webster & Frenkel (346); Brown & Webster (291)]. Lewin (347) examined ultraviolent

induced mutants of *Chlamydomonas* and found one culture with impaired photosynthesis.

The relation between photosynthesis and phosphorus metabolism was thoroughly reviewed in this series last year [Brown & Frenkel (8)]. A few significant papers have subsequently appeared in print, however. Strehler, using the very ingenious firefly luminescence technique for measuring ATP [Strehler & Totter (348)] made careful studies on the ATP content of *Chlorella* as a function of light and other factors (349). Goodman *et al.* (350) examined the distribution of radioactivity in the metabolites of *Scenedesmus* cultured in the presence of radiocative phosphate.

The relation between the phosphate content of plants and photosynthesis was studied by Grube (351) and by Simonis & Grube (352). Haxo & Clendenning (353) made a detailed study of the photosynthesis and phototaxis of the gametes of the alga *Ulva lactuca*. Racusen & Aronoff (354) reported a method for separating quantities of intact parenchyma cells from homogenates of soybean leaves. Suspension sof these cells could carry out photosynthesis at one-fifth the rate of the normal leaf relative to the chlorophyll concentration. The effect of oxygen partial pressure on the photosynthesis of *Bryophyllum* was examined [Moyse (355)]. Withrow *et al.* (356) examined the growth of plants under controlled conditions of temperature, humidity nutrition, and light. The effect of narrow spectral regions in the visibile and far red were examined for effects on pigment synthesis and morphogenesis.

Bacterial photosynthesis.—Gest (357) has recently reviewed the metabolism of photosynthetic bacteria. Kamen and co-workers (358 to 361) have continued their program of study on photosynthetic bacteria. They concluded that no carbon fixation pattern unique to photosynthesis occurs, since they found the same labelling patterns in both the light and dark metabolism of acetate and carbonate by *R. rubrum*. Gest (362) compared the hydrogenase from *R. rubrum* with that from *E. coli* and found them to be very similar. Recently Kamen and co-workers [Vernon & Kamen (221); Vernon (222); Vernon & Kamen (223); Kamen & Vernon (224); Elsden, Kamen & Vernon (225)] have carried out a series of very interesting studies on the participation of cytochromes in the metabolism of photosynthetic bacteria.

For some time it has been noted that the photosynthesis rate versus light intensity curves for purple sulfur bacteria are S-shaped rather than hyperbolic as in most other photosynthetic organisms. This phenomenon has been shown to result from the photochemical hydrogen evolution which occurs along with the normal photosynthetic reaction [Morita *et al.* (363)].

The study of the green photosynthetic bacteria has been reopened by Larsen (364) who isolated new strains of these bacteria including a new species (*Chlorobium thiosulfatophilum*) and compared their metabolism with that of the classical species, *Chlorobium limicola*. The new strains were able

to use thiosulfate and tetrathionate as electron-donors in photosynthesis. The green bacteria were also able to use molecular hydrogen. Larsen *et al.* (365) studied the energy metabolism of the new species of green bacteria described above. The quantum requirement per molecule of carbon dioxide fixed was 10 ± 1 at 732 mμ regardless of the oxidation-reduction potential of the electron donor used (thiosulfate, tetrathionate, molecular hydrogen). These results were regarded as indicating that the primary photochemical reaction in bacterial photosynthesis, as in the green plants, is the photolysis of water. Since the chemical energy produced by oxidation of the electron donor is not utilized for carbon dioxide fixation, the photosynthetic process of the green sulfur bacteria is less efficient thermodynamically than that of the green plants. Pringsheim (366) briefly discussed the taxonomy of the green bacteria. Vinbert & Sivko (367) reported on a type of green bacteria which played an important part in the ecology of certain ponds. This organism allegedly evolved oxygen.

Thomas & Goedherr (368) showed that the light absorbed by the carotenoids of *R. rubrum* was relatively less efficient in phototaxis than in photosynthesis. The action spectrum for the phototaxis of *R. rubrum* was measured quantitatively by Milatz (369). Tsukamoto (370) studied the metabolism of acetate and propionate in both the respiration and photosynthesis of purple bacteria. Ehrensvard & Reio (371) studied the mechanism of synthesis of tyrosine in *R. rubrum* from labelled acetate and carbon dioxide, while Tarver *et al.* (372) studied the biosynthesis of amino acids and protein from labelled carbon dioxide in the same organisms. Abstracts concerning the effect of monofluoroacetate on the metabolism of *R. rubrum* (373), and on the porphyrin synthesis of *Rhodopseudomonas spheroides* (374) appeared.

Photosynthesis and nitrogen fixation.—A clear relationship between photosynthesis and nitrogen fixation was first established by Kamen & Gest (375). They showed that illumination increased the assimilation of molecular nitrogen by the photosynthetic bacterium *R. rubrum*. The general field of nitrogen fixation has been recently reviewed by Wilson (376). However, several significant papers have appeared since. Lindstrom *et al.* (377), using isotopic nitrogen, showed that inoculated plants of *Trifolium pratense* and cultures of *R. rubrum* fixed nitrogen under aerobic conditions in the dark. The rate, however, was markedly increased in the light. Ammonia was identified as a key intermediate in the nitrogen fixation of three types of photosynthetic bacteria (*Chromatium, Chlorobacterium,* and *R. rubrum*) by Wall *et al.* (378). Newton *et al.* (379) investigated the nitrogen fixation and hydrogen production of additional strains of *Chromatium*. Pure cultures of the blue-green alga *Nostoc commune* were shown to fix atmospheric nitrogen on illumination [Herisset (380)]. This reaction was inhibited by the presence of ammonium salts or nitrate. Fogg, in an extensive paper (381), examined the nature of the extracellular nitrogenous substances produced by the nitro-

gen-fixing blue-green alga *Anabaena cylindrica*. Culture conditions influenc-
ing production were also investigated.

    Photosynthesis under natural and field conditions.—Verduin continued his
studies of the photosynthesis of aquatic plants under more or less natural
conditions (382 to 384). He also prepared an excellent review of the photo-
synthetic rates under optimal, near-natural conditions of many types of
plants including photosynthetic bacteria, algae, mosses and lichens, water
plants, desert plants, alpine plants, arctic plants, tropical and subtropical
trees and shrubs as well as temperate zone plants of various types [Verduin
(385)]. A survey of the summer plankton productivity of a fresh water lake
was made by Tryon & Jackson (386), while Sargent & Lantrip (387) made
simultaneous measurements on the photosynthesis and growth rate of the
giant kelp *Macrocystis pyrifera*.

    Tombesi & Fortini (388) examined the effect of reduced water supply
on the photosynthesis, respiration, and enzyme activity of the geranium.
Tombesi *et al.* (389) examined the changes in photosynthesis, respiration,
enzyme properties, etc., of the leaves of a number of plants during growth,
flowering, and ripening. Brilliant & Krupnikova (390) examined photosyn-
thesis in detached leaves as a function of temperature and degree of dehydra-
tion. Briganti (391) examined the adaptation to light of the photosynthesis
of wheat, tobacco, and *Elodea* plants which had been kept in the dark.
Freeland (392) examined the photosynthetic rates of the leaves of a number
of conifers as a function of age. Leaves of all species examined showed their
maximum rates during the first year, but leaves which were three years old
still showed measurable photosynthesis.

    Algae as food.—As Spoehr (393) has pointed out, the problem of a satis-
factory supply of food is an old one, as old as man himself. In recent years
the problem has assumed great importance because of the rapidly increasing
world population and the relatively slow rate of putting new areas under
cultivation. One possible solution of the problem lies in developing plants
capable of utilizing solar energy more efficiently for the synthesis of organic
compounds. It is well known that *Chlorella* and certain other unicellular
algae can carry out this process under optimum laboratory conditions with
a very high efficiency compared to ordinary agricultural crops raised under
field conditions. As a result, a number of workers have investigated the possi-
bilities of using *Chlorella* or other algae as a source of food and chemicals.
The most recent work is based on a broad foundation of earlier investigations
by a number of workers on algal physiology which cannot be reviewed here.

    Milner (394) made a brief report on some of the problems involved in
mass culture of *Chlorella*, and Woodward (395) reviewed the possible uses of
Chlorella as a source of food and chemicals. A number of studies have been
made on the food value of *Chlorella*. Fowden (396) examined the bulk protein
of *Chlorella*. It was low in cystine, but the essential amino acids made up over

50 per cent of the total weight of amino acids. Combs (397) examined dried *Chlorella* as a source of food for the chick and found, except for mechanical difficulties in handling the material, that it could serve as a source of certain necessary nutrients. Schieler *et al.* (398) have recently made analyses of the amino acid composition of *Chlorella pyrenoidosa* and *C. vulgaris* protein. They concluded that *Chlorella* protein (and possibly the cells) could serve as a satisfactory amino acid source for animals if supplemented with cystine, methionine and histidine. Beresford (399) has investigated the culture conditions necessary for maximum fat production by green algae.

The Carnegie Institution of Washington supported and encouraged an extensive program of study on the cultivation of algae as a source of food which culminated in a pilot-plant experiment carried out by Arthur D. Little, Inc. The culture unit consisted of a 160 ft. length of polyethylene tubing four feet wide and a few inches high which held approximately 1200 gallons of culture solution. This unit, using natural sunlight, produced about one pound dry weight of *Chlorella* per day or, roughly, 16 tons per acre per year. This work culminated in a publication by the Carnegie Institution [Burlew, J. S., editor (400)] consisting of 22 papers which cover all aspects of algae culture. A selected list of references relating to mass culture of algae is also included.

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OCCURRENCE, FORMATION, AND INACTIVATION OF AUXINS[1,2]

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Since the last review of this topic by Larsen (136), pertinent material has appeared in related reviews (31, 75, 163, 164, 181, 234, 241) and in books devoted entirely to plant growth substances (10, 205, 211). Auxin production by crown gall bacteria and host tissues, and its relation to tumor development, has been reviewed recently by Klein (118). Emphasis is placed in this discussion on auxins and related compounds known to occur in plants. Rather than attempting to secure complete coverage, the aim is to present an interpretation of the present status of auxin metabolism and related *in vitro* phenomena.

Terminology.—Efforts have been made recently to reduce the confusion resulting from lack of uniformity in growth-substance terminology (75, Committee[3] on Nomenclature of Plant Regulators, American Society of Plant Physiologists). The results afford a workable and needed compromise until more precisely defined criteria are available. In accordance with the proposed usages, growth substances or growth regulators are organic compounds, other than nutrients, which in small amounts modify growth through alteration of physiological processes (cf. also 252). Auxin will be considered as a generic term describing those growth regulators which, though of multiple effect, have the specific ability to stimulate cellular enlargement in shoots. The definition depends essentially upon biological assay as a criterion, usually straight growth of shoots or shoot segments, or curvature induced by differential response of opposing tissues. Naturally occurring auxins shown to possess a correlative function may be designated as hormones. Chemical structures characteristic of the auxins are, or are analogues of, aromatic carbocyclic or heterocyclic ring systems with a side chain bearing a carboxyl group. Many of the compounds commonly considered as growth substances, although not auxins, may be so described structurally.

The extensive literature on the relation of structure to physiological activity has recently been reviewed (241; cf. also 1, 2, 3, 31, 151, 159). These discuss the structural modifications which produce antiauxin activity, or auxin antagonism. McRae, Foster & Bonner (150) defined an antiauxin as a

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[1] The survey of the literature pertaining to this review was completed in December, 1953.

[2] The following abbreviations will be used: IAA, indoleacetic acid; IAc, indoleacetaldehyde; IAN, indoleacetonitrile; IPyA, indolepyruvic acid; ET·IAA, ethylindoleacetate; NAA, naphthaleneacetic acid; NAc, naphthaleneacetaldehyde; ATP, adenosinetriphosphate; 2-4-D, dichlorophenoxyacetic acid.

[3] J. van Overbeek (Chairman), P. Larsen, R. M. Muir, H. B. Tukey, F. W. Went.

"substance not itself possessed of growth-promoting activity but with the ability to inhibit competititvely the action of auxins." Since weak auxins (31) and indeed, active auxins themselves (150) may competitively interact to result in net growth reductions, it would be preferable to consider anti-auxins as "compounds which inhibit competitively the action of auxins."[3] Yet, substances which may depress auxin-induced growth indirectly (see below) have been likewise termed antiauxins. Such indirect action is often not clearly discernible, particularly with compounds bearing some obvious structural relation to auxins. Hence, it may be desirable at this stage simply to group compounds inhibiting auxin action, whose effects are to some degree reversible by auxin, as auxin antagonists (cf. 31). The qualification, competitive or noncompetitive, may then be employed as indicated experimentally by inhibition indices or kinetic studies (267).

When dealing with the state of auxin in the plant, free auxin may be considered as the auxin readily and quickly extracted from the plant by methods involving minimal comversion or interconversion of cellular components. Rapid cold ether extraction (172), lyophylization and low temperature extraction (263), and short term diffusion (253) appear to be useful in yielding estimates of free auxin levels. Though the data are scanty, rapid drying of plant material under infrared lamps and forced draft appears to yield similar results to the lyophylization technique (45).

Bound auxin may be considered as auxin immobilized by linkage to other molecules regardless of the nature of the bond involved. It may also refer to auxin no longer extractable in a form active on bioassay. Implicit is the connotation that the structure of the auxin molecule is to a large extent retained. There appears to be no evidence that such auxin-complexes normally function as auxin precursors *in vivo*. The term "auxin precursor" will be restricted to compounds which are converted to auxin by plant tissues or tissue fractions. Again, such conversions do not necessarily indicate that the precursor normally occurs or functions as a source of auxin in the plant.

Some of the methods so far used to evaluate auxin production, or the reserve supply of potentially available auxin, are of debatable value in terms of physiological applicability. Many tissues under nonpolar solvents will continue to yield auxin for extended periods of time, even years (234). It is highly questionable whether extraction techniques which permit autolytic conversions represent the normal interactions of cellular components (cf. Went in 204). Tissue hydrolyses by enzymes or alkali, while they may indicate bound forms of auxin, or possible auxin precursors, have yielded little information so far in regard to either auxin production or function. As has been pointed out (75), prolonged or exhaustive diffusion as an indication of auxin production has the advantage of maintaining the organ or tissue essentially intact, and allowing the biochemical relations within the tissues to approximate more closely their normal state. Yet even this technique cannot be accepted without qualification. Depending on the movement of auxin from a tissue to an aqueous receptor or receptor tissue by an auxin concentra-

tion gradient, results are affected by the transport capacity of the tissue to the diffusion interface, as well as by enzymatic oxidation of auxin at the interface (229, 253, 257). In addition, continued formation of auxin in an organ or tissue may depend on the supply of precursors normally transported to the material examined from other sites in the intact plant or seed. Such a dependence seems likely for the embryo (163, 268), buds and coleoptile tips (253) and probably other meristems. Nevertheless, the diffusion technique has proven of value in gauging the relative ability of various plant parts to produce auxin, and many of the now classical concepts of hormonal production and destruction have derived from its use (253). As will be indicated, commonly postulated sites of auxin production show relatively high activities in the conversion of potentially native auxin precursors.

OCCURRENCE AND IDENTIFICATION

It is becoming clear that IAA[2] is an auxin which occurs in most if not all plants. Also responsible for the emphasis on IAA in physiological interpretations has been lack of substantiation for the occurrence of auxins *a* and *b*. One of the main difficulties in the sphere of auxin identification is the minute concentrations at which auxin is found in most plant tissues. Direct isolation and characterization is usually impractical. Biological assays for auxin are, as a whole, nonspecific, resulting in the dependence on indirect criteria for identification. It was a first considered that the auxins native to plants were either auxins *a*, *b* or IAA. These possessed different molecular weights and labilities to hot acid and alkali. Identification of native auxins therefore relied heavily on determination of the acid-alkali sensitivity and diffusion constant of the biological activity in plant extracts. Many plant extracts showed a sensitivity to acid, a relative stability to alkali, and a diffusion velocity in agar similar to pure IAA. Other results indicated the presence of auxin apparently stable in acid, or having diffusion velocities yielding molecular weights well above that for IAA. (For summaries, cf. 126, 131, 136, 211.)

Additional indirect tests also have been used for identification of the auxin in gross extracts. The modified Salkowski color reagent, in which IAA reacts with iron in mineral acid (155, 220) to yield a chromophore with a band near 530 mμ, was used to identify the major auxin in *Avena* coleoptiles as IAA (260). The disappearance of auxin activity in extracts after treatment with IAA-oxidase (220) preparations has been interpreted to indicate that the auxin was IAA (77, 157, 158, 191). Since the concentration-response curves in a bioassay are characteristic for different pure auxins, response curves of pure IAA and extract activity when adjusted to one common value have been considered as support or nonsupport of identity (77, 100, 131, 158, 191). To these may be added other differences in plant response and transport behavior between pure IAA and plant extracts (cf. in particular, 87, 88).

Some of the uncertainties involved in using these various criteria for

auxin identification have been discussed by Larsen (136) and further elabo-
rated upon in (75). Perhaps most important is that the tests are of doubtful
specificity; moreover, the results obtained can be markedly influenced by the
nonauxin components present in impure extracts. Even the disappearance of
auxin activity after treatment with crude IAA-oxidase preparations cannot
be accepted as definitive for IAA. The degree of specificity assigned to this
enzyme rests chiefly on: the lack of disappearance of Hopkins-Cole reagent
color with other indole analogues (220), the O_2 uptake with higher homo-
logues (247), and the virtually unaltered rates of IAA destruction in the
presence of various synthetic auxins (220)—all determined at relatively
high substrate levels. This does not preclude the disappearance of auxin-
activity in biological extracts, where the auxin content is usually low, when
treated with crude IAA-oxidase by other, nonspecific mechanisms: protein
binding (200, 207, 208, 210), CoA esterfication (138), or the action of con-
taminating enzymes. Furthermore, several compounds with auxin activity,
other than IAA and auxins a and b, may occur in plant extracts. At most,
then, the indirect tests mentioned above should be considered only as sup-
plementary adjuncts in auxin identification.

The problem of auxin occurrence and identification in biological extracts
has been recently clarified by techniques yielding less equivocal results.
Foremost among these has been differential migration analysis. Counter
current distribution has also been used. Holley *et al.* (104) used the latter
method with the ether-soluble acidic fraction of cabbage leaves. The major
active fraction possessed the distribution characteristic of IAA, giving a
color density with the iron-sulfuric acid reagent (252) corresponding to its
activity by bioassay. Two aditional components with auxin activity were
likewise found. [Pertinent to the low specificity of the color reagent, the
latter components, as well as fractions devoid of biological activity, also
yielded appreciable colors (cf. also 80, 217). It is likewise of interest that two
growth substances have been reportèd (145) for *Brassica* which gave negative
indole color reactions.]

Several types of differential migration have been employed for auxin
separation in the past three years: paper chromatography (12, 18, 19, 29a,
47, 110, 140, 147, 173, 217, 227, 228, 244, 266, 268), paper ionophoresis (46,
47), and column chromatography (29b, 46, 54, 84, 101, 144, 145). After res-
olution, both bioasssay and color reagents have been utilized for auxin
location. Most of the color reagents used react with indole derivatives: ferric
chloride in sulfuric acid (220), in hydrochloric acid (268), or in perchloric
acid (19, 217); Ehrlich's p-dimethylaminobenzaldehyde (173, 217, 244);
Wieland's cinnamic aldehyde (110, 227); nitrite (46, 54), diazotized sulfanilic
acid and p-nitroaniline (217); and Van Eck's benzidine for IAc[2] (268). Some
of the compounds identified by chromatography of biological extracts are
summarized in Table I. They comprise IAA, IAc, Et·IAA, and IAN.[2]

Perhaps the most extensive investigation on the paper chromatography
of IAA and analogues was carried out by Stowe & Thimann (216, 217). They
dertermined the chromatographic behavior and color reactions of 35 indolyl

TABLE I

AUXINS AND RELATED COMPOUNDS IN BIOLOGICAL EXTRACTS IDENTIFIED
BY CHROMATOGRAPHIC METHODS

Material	Extract	Method	Delimitation	Indicated	Ref.
Pea seedlings	Et₂O	P	B	IAA	12
Various: Shoots				IAA	
Roots				one[b]	
Potato tuber	EtOH[a]	P	B, C	IAN in tuber	18
Aegopodium rhizome				and rhizome	
Sunflower seedling	Et₂O[a]	P	B	IAA	19
Urine	Et₂O	P	B	IAA	19
Hevea leaves	Et₂O	P	B	IAA	29a
Cabbage leaves	EtOH	P, S, I	C, B	IAN	46
				Indolealdehyde two[b]	
Cabbage leaves	H₂O	S	C	IAN	54
				Indolealdehyde	
Cabbage leaves	CCl₄	S	Isol. & Char.	IAN	101
Wheat roots	Et₂O[a]	P	B	IAA one[b]	140
Broccoli	EtOH	S	B, C	IAA	144
Brassica	EtOH	S	B, C	four[b]	145
Asparagus fruit and Broccoli leaves	{Et₂O water}	P	B	IAA	147
Various organs	{Et₂O water}	P	B	one[b]	147
Potato tuber	Pancreatin digest[a]	P	C	IAA	173
Corn kernels	Aqu. Acetone	P	C, B	IPyA IAA two[b]	217
Oat coleoptiles	Et₂O Et₂O[a] Water	P	B	IAA	227
Apple endosperm	Et₂O	P	C	Et·IAA	228
Barley shoots Tomato shoots	alk. hydrolysate	P	C	IAA	244
Corn kernels	alk. hydrolysate of acetone extract	P	C	IAA	244
Corn smut	Et₂O	P	C	IAA	266
Corn kernels	Et₂O	P	C, B	IAA IAc	268
Corn kernels	H₂O	P	C	Tryptophan	268

P = Paper, S = Column, I = Ionophoresis, B = Bioassay, C = Color reagent,
[a] = acidic fraction.   [b] = not identified.

compounds, varying the solvents and pH, and gave the chromophore absorption maxima in the visible spectrum of 21 compounds treated with the iron-perchloric acid reagent. Such absorption data should be valuable as an additional aid in delimitation. A mixture of isopropanol 8 parts: 28 per cent aqueous ammonia 1 part: water 1 part by volume is suggested as one of the most versatile of the solvent systems. In 1944 Berger & Avery (24) isolated and described the properties of an auxin "precursor" obtained from dormant sweet corn kernels (Country Gentleman). Possessing low auxin activity, it liberated large quantities of auxin upon alkaline hydrolysis. IAA was crystallized from the alkaline hydrolysate, although infrared absorption spectra showed an appreciable carbonyl contaminant. The complex was again extracted from corn kernels by Stowe and Thinmann following the original procedure of Berger and Avery, and by rapid acetone extraction at low temperature. On chromatography, both before and after alkaline hydrolysis, four components with auxin activity appeared. Two were identified as IPyA[2] and IAA on the basis of R_f and color reactions. This is the first reported instance of IPyA occurring in higher plants. The two unidentified auxins, though probably indolyl compounds, could not be identified as IAN, IAc or Et·IAA on the basis of R_f and color reactions. One, however, like pure IAN (21, 235), appeared to have low or no activity in the pea test but was active when tested by *Avena* sections. The finding of IPyA, which moves more slowly than IAA when developed as the salt, suggested to Stowe and Thimann the possible like identity of components noted by other workers. With similar techniques, slower moving acidic substances were observed in extracts of various plants by Bennett-Clark & Kefford (18), by Lexander with wheat roots (140), and by Denffer, *et al.* with cabbage leaves (46).

Though the occurrence of IAA in corn kernels seems established (91, 217, 268), some lack of agreement in the identity of other indolyl compounds observed in this material is worth comment. Et·IAA was isolated and characterized by Redemann *et al.* (190) from cold ethanolic extracts of immature Golden Cross kernels. The unidentified auxin with an R_f of about .83 in water saturated butanol-ammonia, which Luckwill (147) found in apple endosperm (as well as certain buds, seeds, stamens, stem tips, and leaves), was identified by Teubner (228) as Et·IAA. Moreover, considerable yields of auxin were obtained on hydrolysis of a number of seed oils by lipase as well as alkali (121). Yet no indication of the ester's presence was found in the chromatographic studies with extracts of Country Gentleman (217) and probably not with those of Silver Bantam (268) kernels. Similarly, IPyA and the other two unidentified auxins found in the Country Gentleman variety were not observed in like extracts of another variety (217) or in Golden Cross Bantam (244). And although IAc was identified in Silver Bantam (268), it did not appear to occur in Country Gentleman (217). Such disparities may be attributable (217) to varietal differences in corn, where appreciable differences in auxin levels (13, 223) and extent of destruction (168, 169) have

been observed, though one would anticipate quantitative rather than qualitative differences in the auxins and related compounds of a given species.

Several additional considerations are pertinent to the determination of occurrence or nonoccurrence of the various compounds so far identified. They relate to compound lability, extraction method, and concentration in the plant. First, IPyA, like most α-keto acids, is labile and breaks down in aqueous solution to both IAA and possibly IAc, as well as other products (77, 217, 261). The reaction appears to be accelerated at both acid and alkaline pH (96, 261). IAc (or what may be presumed to be IAc) is sensitive to alkali and even more so to acid (77, 100, 131). Like other aryl-acetaldehydes (111, 116), it tends to polymerize or condense (E. R. Jones, personal communication), even in solution. There are definite indications that oxidation of IAA occurs on chromatographic strips exposed to the air or in pigmented solutions exposed to the light (110, 145, 219). Chromatograms of synthetic C^{14} labeled IAA (219) showed two additional spots on radioautography when drying and development of the applied sample was carried on in air. These secondary spots were absent or greatly diminished when manipulations were performed under nitrogen. Second, the extraction time and solvent used with the plant material may influence the results obtained, and raises the issue of possible artifacts. The comments made before concerning free auxin extraction methods seem applicable here. Even the use of ethanol as a solvent was questioned by Henbest, *et al.* (101), who suggested that the Et·IAA isolated from corn kernels (190) might be an esterification artifact formed during ethanol extraction. Though no Et·IAA was obtained by treatment of IAA with ethanol under conditions similar to those used for isolation of the ester (190), this is not exactly analogous to the treatment of corn kernels. It is also obvious that the type of compounds extracted will be influenced by the solvent used. Third, the fact that no IAA was detected at its expected R_f in chromatographs of some plant materials where color reaction of the sprayed sheet was used as the sole criterion (228, 244) is not necessarily an indication of the absence of this auxin. In many plant tissues the concentrations of IAA and related molecules are sufficiently low so as to be below the threshold of detection by chromophore. For example, bioassay of strip segments enabled Audus & Thresh (12) to estimate the IAA concentration in pea seedlings as roughly 2×10^{-4} μg./gm. tissue. Such concentrations would require sample sizes in the order of kilograms to come into significant detection range of most color reagents.

The work of Yamaki & Nakamura (268) provides an additional indication of the occurrence of IAc. Germinated corn seeds were extracted with ether and the neutral fraction treated with sodium bisulfite. The properties of the regenerated adduct, purified by vacuum sublimation, were identical with those of the preparation, presumably IAc, synthesized by the tryptophan-isatin reaction (7, 131). Both preparations were similarly active on bioassay, convertible to IAA by raw milk, labile to hot alkali and acid,

and relatively stable at neutrality. Their R_f and benzidine reactions were the same, and both formed adduncts with bisulfite and dimedone. The above cited work supplements the evidence, reviewed in (136) that a neutral component in many plant extracts is IAc. In addition, IAA was more conclusively identified by paper chromatography as the acid auxin formed from IAc preparations by the action of corn embryo juice. In brief, the case for the naturally occurring neutral component being IAc rests on the following observations: the apparent molecular weight as indicated by diffusion velocity; its enzymatic conversion of IAA by plants, plant fractions, and aldehydrase preparations (unfortunately, not enzymatically homogeneous); perhaps least equivocal, is its formation of a regeneratable bisulfite addition compound, and its reactivity with the more specific aldehyde reagent dimedone. Moreover, the neutral product of the reaction of ninhydrin or isatin with tryptophan is virtually identical with the natural substance in the above characteristics. Though the ninhydrin reaction will yield the lower corresponding aldehyde with many amino acids (193), it is of interest that Virtanen *et al.* (243) found that no significant amount of volatile aldehyde was formed with tryptophan. This observation is not inconsistent with the low yields of IAc obtained (7, 131) in this reaction.

Pure IAc has now been synthesized and characterized more rigorously (38, 39) by absorption spectra and as the semicarbazone, dinitrophenyl-hydrazone, and dimedone derivatives. The activities of the synthetic aldehyde in the *Avena* coleoptile section test, the pea test, and in a modified (21) Moewus cress root test have also been determined (22). With coleptile sections, IAc exhibits an activity of about 1/4, 1/11, and 1/55 of IAA at aldehyde solution concentrations, respectively, of 0.1, 1.0 and 10 mg./l. In the pea and cress tests, IAc is reported (22) about 5 to 10 times less active than IAA over a range of concentrations from 0.01 to 10 mg./l. Though not directly comparable, these apparent relative activities found for the aldehyde by Bentley & Housley (22) are in the same order as those previously deduced by Larsen (133, 134, 135) with the *Avena* curvature test. The above characterizations of pure IAc should facilitate the highly desirable demonstration on stricter chemical grounds of this aldehyde's occurrence in plants.

The presence of a neutral growth promoting substance in a plant extract cannot, *ipso facto*, be attributed necessarily or solely to IAc. In addition to Et·IAA, the possibility of IAN as an active component must now be recognized. Very large auxin activities in extracts of various *Brassica* had been observed by several workers (16, 142, 143) and this high activity was found to reside largely in the neutral fraction of extracts of a number of the Cruciferae (101, 113). With emphasis on free auxin extraction methods, Jones and co-workers (101, 113) demonstrated the occurrence of IAN in cabbage by isolation in crystallin form and by proof of structure. This is the first auxin from vegetative tissues of higher plants to be so characterized. Chromatographic studies have also identified IAN in cabbage leaves (46, 54) and in the

tuber and rhizome (18). Unlike IAA, the nitrile is apparently stable to hot N acid in the presence or absence of O_2 (101). It is labile in hot N alkali, being in part hydrolyzed to IAA (22, 101), and will not form a bisulfite adduct or semicarbazone (101).

Bentley *et al*. (21, 22, 23) have examined the biological behavior of IAN and shown that it possesses some, though not all, of the activities commonly ascribed to the auxins. IAN at concentrations below 10 mg./l. is more active than IAA in enhancing growth of *Avena* coleoptile sections (22, 23), probably because of greater tissue penetrability, though the nitrile is less inhibitory at high concentrations. In the *Avena* curvature test, however, IAN is less active than the acid. This is quite likely caused by greater lateral spread of the nitrile across the coleoptile when applied unilaterally in the curvature test, the growth on both sides causing a reduction in curvature (21). Surprisingly, IAN is inactive in the pea test (21, 167, 235, 239) until concentrations of about 50 mg./l. are used, where slight activity appears (21, 167). Thimann (235) also found the nitrile to be inactive in straight growth of pea sections, and only slightly active with slit corn coleoptiles and *Lupinus* hypocotls. The activity of IAc in both the *Avena* section and pea tests, and inactivity of IAN in the latter, suggests the concomitant use of these tests to differentiate between the nitrile and aldehyde in plant extracts (21). In the pea test, however, concentrations of IAN, inactive *per se*, significantly enhance the response to IAA (167), an action which was reported not to occur in the *Avena* coleoptile (22). The apparent transport of IAN in the *Avena* coleoptile is identical with IAA (21), i.e., strictly basipolar. Finally, the nitrile was about as active as the acid or somewhat less so in the initiation of cambial activity, in the inhibition of root growth, and in induction of parthenocarpy (where the response to the nitrile was delayed). Unlike IAA, it was virtually inactive in root initiation and in the inhibition of lateral buds and petiole abcission. As Bentley & Bickle suggest (21), it would be desirable to determine the occurrence and distribution of IAN in the tissues used in the various tests, particularly those where the nitrile is inactive.

It seems also unwarranted to assume that the auxin activity of the neutral fraction of various plant extracts is caused solely by IAN. The nitrile certainly accounts for a major part of the activity in members of the Cruciferae (101), and evidence is presented (21) which suggests that the radish "inhibitor" studied by Stewart (215) was IAN. However, evidence for the occurrence of the nitrile in other families is scanty so far (18). In this connection, some recent results (251) obtained with cabbage leaf extracts are difficult to reconcile with the idea that the nitrile was the sole active neutral component. As might be anticipated, pure IAN did not react with dimedone, the activity of the nitrile in the *Avena* curvature test being unaffected by previous incubation with the aldehyde reagent. Similarly, no reaction with dimedone was observed in suitably controlled experiments where the nitrile plus the neutral fraction of a cabbage ether extract was treated with the reagent. Yet the direct activity in the curvature test of the neutral fraction

from both rapid as well as prolonged ether extractions of cabbage was reduced by pretreatment with dimedone. In addition, similar pretreatment of the cabbage neutral fractions sharply reduced the relatively large amount of acid auxin normally formed by incubation of the neutral fractions with coleoptile juice (see below). Such results are certainly suggestive of the presence of IAc in cabbage neutral fractions.

It is evident that a number of compounds with auxin activity occur in plants. The preponderance of observations indicate the active substances to be either IAA or other indolyl compounds. IAA (91, 123, 230), IAN (101), Et·IAA (190), tryptophan, and tryptamine (258) have been definitely identified. The occurrence of IAc and IPyA may be considered as highly probable short of isolation as the pure compounds. That is not to say that these compounds are the only ones native to plants with auxin activity. Some of the unidentified substances that are listed in Table I, as well as others (17, 49 158, 179, 180), may very well fall into the above group. The cited work of Stowe & Thimann (217) is particularly suggestive of other active substances, while Sen & Leopold (196) have reported the chromatographic separation of an auxin from barley leaves that appears to be an indoxyl compound. Phenylacetic and *cis*-cinnamic acids and their esters which exhibit, respectively, weak and strong auxin activities (253), are known to occur in plants [Haagen-Smit in (205)]. However, no information is available as yet whereby a physiological role for these compounds as auxins may be adduced. More certain, moreover, is the hormonal function of IAA. Pure IAA has the polar pattern of transport (212) and correlative action as the native auxin formed at production loci (234). Recent studies in tropisms (34, 36, 129) and in the histogenic action of IAA (42, 109, 256, 264) continue to confirm the generalization.

Assuming that primary auxin activity is a characteristic of IAA and its various ring analogues [Thimann in (205)], the question is whether the indolyl analogues of IAA so far identified in plants are active as such, or manifest activity as precursors of auxin. We encounter here the weakness of the definition of auxins, whose criterion of cellular elongation does not readily distinguish between directly active compounds and those indirectly active via tissue conversion. A lag in tissue or organ response to a substance, or the absence of a lag period, is not necessarily conclusive, since rapidity of response may be determined by the rates of tissue absorption and entry into reactive cells. Lack of knowledge of the auxin action mechanism prevents a clear distinction between direct and indirect action. Practically, a precursor function may be presumed if it can be shown that the positive auxin response elicited by a compound is accompanied by the formation of an acid to which direct activity may be attributed, or if a like formation is readily accomplished by tissue fractions. The present ideas of IAA biogenesis have developed by essentially such an approach. As will be indicated in the following section, the auxin activity exhibited by several of the naturally occurring indolyl compounds may be ascribed to their conversion to IAA.

FORMATION

Larsen (136) had comprehensively reviewed the literature to 1951 pertaining to the biochemistry of IAA formation in plant tissues. The above work may be summarized in the frequently encountered (10, 30, 75, 77, 114, 136, 197) formulation of IAA origin: IAc arises from tryptophan as a primary precursor, via either IPyA or tryptamine, the aldehyde being then oxidized to IAA. The evidence concerning this scheme will be briefly discussed and supplemented by more recent observations.

The amino acid.—Though tryptophan is inactive in the standard *Avena* curvature test (141, 202), it yields a delayed response in this test (202). However, more direct contiguity between tryptophan and either tissues or plant enzyme preparations clearly established that many plants and plant organs can convert the amino acid to directly active auxin. This capacity is possessed by the leaf, apical bud, epicotyl, stem, coleoptile, ovary, endosperm and cotyledons, crown gall and callous tissue, and by bacteria and fungi. To these may be added the embryo (268), pollen (163), tissue cultures (128; but see 166) and possibly roots (11). While it is quite probable that the acid auxin produced is IAA (77) this has been only shown rigorously by isolation from *Rhizopus* (230) and indicated by chromatography with corn embryos (268) and corn smut (266; cf. 90). The tryptophan conversion has a pH optimum of about 7 to 8 (102, 261). It apparently requires oxygen (230, 261) and is inhibited by cyanide (261, 263) and bisulfite (261).

Indications that auxin biosynthesis involves tryptophan derive mainly from correlated phenomena. The yield of IAA in *Rhizopus* cultures was observed to depend upon the tryptophan content of the media (230). Most efficient conversions of the amino acid to auxin are accomplished by those tissues or organs known to function as auxin production loci. Fertilized ovaries are relatively high in conversion efficiency (263), mature leaves are less effective than young leaves and buds (76, 251), and the coleoptile tip is about six times as effective as the remainder of the coleoptile on a tissue-weight basis (260). The parallelism still holds for tryptophan as a potential substrate. As one proceeds from mature to young or more metabolically active organs in the green plant, the tryptophan concentration increases (165, 238) in a pattern similar to that found for auxin distribution (86, 170, 237). Similar correlations may be drawn for the achenes of the strawberry (163), for certain crown gall bacteria (118), and for corn kernels (222, 223). During the growth of germinating corn embryos, either excised or intact, their levels of both IAc and IAA were found to vary in the same manner as the endogenous level of tryptophan (268). At the same time, the levels of IAc and IAA in the endosperm were relatively low and remained essentially constant. The inference could be drawn that auxin production in the embryos was in part controlled by the availability of tryptophan in a highly competitive system for tryptophan utilization. A similar inference is possible from the work of Tsui (238). The low auxin content of Zn-deficient tomato plants (203, 238) was clearly associated with the decreased levels of tryptophan re-

sulting from the mineral deficiency. It should be realized, however, that several key enzyme systems in plants are profoundly affected by Zn deficiency, viz., aldolase (185), alcohol dehydrogenase, and desoxyribonuclease (160).

Experiments on the effect of ionizing radiation upon auxin economy also tend to substantiate the concept that IAA is normally formed from tryptophan. Auxin production in plant tissues is inhibited by low doses of ionizing radiation (78, 201). If the radiation dose is not too high, normal levels of production are reattained (78). The enzymes converting trytophan to IAA were found to have *in vivo* radiosensitivities and regeneratability patterns virtually identical with those of auxin formation by the plant (251).

The aldehyde.—IAc prepared by the tryptophan-isatin reaction is readily converted to acid auxin by coleoptiles and coleoptile juice (133) but not, apparently, by cress roots (178). The acid produced by coleoptiles may be presumed to be IAA, an assumption strengthened by the similarity of concentration-response curves obtained (133). The regenerated bisulfite addition product of pineapple and dandelion roots, which reacts with dimedone, is likewise converted to acid auxin by pineapple leaf breis and a partially purified enzyme prepared from the breis (77). Corn embryo juice will also form the active acid, identified as IAA by R_f, from the regenerated bisulfite adduct of corn (268). Finally, coleoptile sections immersed in solutions of pure IAc were found to produce an acid auxin (22). All of the auxin activity exhibited by the authentic aldehyde could be accounted for by assuming the acid auxin produced was IAA, thus substantiating the previous deduction of Larsen (133) that the auxin activity of IAc results from its conversion to IAA.

There are likewise indications that some synthetic aldehyde analogues of IAc owe their auxin activity to conversion *in vivo* to the corresponding acid (8), and for certain other aldehydes (20, 115, 233, 240, 270) such conversion may be inferred. Synthetic NAc (111, 112) exhibits weak activity in the *Avena* curvature test (132, 135) and an activity approaching that of its acid in the pea test (242). It is rapidly converted to an active acid, probably NAA, by coleoptile juice (134, 135) and by *Artemisia* root juice (9). A dismutation was suggested by Larsen (135) as the mechanism of NAc transformation by coleoptile juice on the basis that two moles of aldehyde disappeared per mole of active acid formed. By analogy, a mutase action upon IAc would yield tryptophol. The alchol is virtually inactive in the *Avena* curvature test (132) and but slightly active in the pea test (242). A mutase active on arylaldehydes has been found in sprouting peas (153). Ashby (7), on the other hand, in the conversion of NAc to NAA by *Artemisia* root juice, found considerably less amounts of the acid than would be anticipated by an aldehyde dismutation. Since NAA was stable in the root juice, he suggested the presence of a factor able to change the aldehyde in some manner other than by the oxidation or dismutation that yields acid auxin. This factor may very well have been an aldehyde oxidase recently described (116).

Kenten (116) showed that the saps of both aerial and underground organs of a number of plants contained a phenylacetaldehyde oxidase system. Since it will also oxidize NAc, a similar action on IAc is highly probable. Pea root sap was particularly active, the stem possessing about 2/3 and the leaf about 1/3 of the root activity. The major action of this enzyme system was shown to consist of the oxidative degradation of phenylacetaldehyde with the formation of benzaldehyde and formic acid, though other products, including phenylacetic acid, are probably likewise formed. Convincing evidence is presented that the major action of the enzyme is that of a peroxidase-metal coupled oxidation system. The occurrence of this system is of particular interest in regard to the conversion of aryl-acetaldehydes to their corresponding acids in plant tissues or by plant extracts. The absolute acid yields obtained, except with coleoptile juice (135), are generally low (7, 22, 38, 77, 131, 268), even considering the losses entailed by fractionation and preparation for bioassay in some of the experiments. It is entirely possible that competition for available aldehyde by the above oxidase was a contributing factor, though with IAc, low acid yields through IAA-oxidase action must also be considered. In the same vein, the half-mole yields of active acid from NAc observed by Larsen (135) could equally have resulted from the presence of both the degradative oxidase and an aldehydrase, as well as from a "mutase" (cf. 189) action. It is clear that the nature of the enzyme catalyzing the conversion of the aldehydes to the corresponding active acids remains undefined. In view of the above discussion, the characterization would probably be more satisfactorily approached by using formation of the acid as a major index in resolution, rather than aldehyde disappearance or oxidation.

Of the organ saps examined, Kenten (116) observed that the root of the dandelion possessed negligible activity (O_2 uptake) in the oxidation of phenylacetaldehyde. This may explain the high levels of IAc found to occur in the roots of the same species (77). An additional implication of the enzyme's widespread occurrence (116) may be drawn. If we assume the presence of IAc in plants, the occurrence of 3-indolealdehyde would be anticipated, though other potential pathways of origin may exist (see below). It is of interest, therefore, that indolealdehyde has been indirectly identified as a component of cabbage extracts by differential migration rates, adsorption behavior, and color reaction (46, 54).

Evidence of IAc function in normal auxin production is weakened by the fact that prolonged rather than rapid ether extractions were used (77, 250, 268) to determine changes in level of the neutral component. Short-time extractions at low temperatures remove inappreciable amounts of IAc from many tissues (77, 137). In *Cichorium* root cuttings, an increase in the neutral form at the distal ends paralleled, but preceded, the increase in free acid auxin (250). In the pineapple plant, the capacity to form the aldehyde decreased with increasing morphological maturity of the tissue examined (77). Various parts of the pineapple plant, after removal of the

free auxin, show a high correlation between the amounts of IAc and IAA
that were subsequently produced (77). A similar parallelism was found for
the corn embryo at different stages of growth (268). However, the rate of
IAA formation in pineapple leaf breis appeared to depend on the rate at
which the aldehyde was produced (77). Moreover, the auxin yield from these
breis fell to negligible levels in the presence of dimedone. Finally, the yields
of IAc from etiolated pea plants were high and IAA low, whereas the rela-
tive levels of each were reversed in comparable light-grown material (131).
The same inverse relationship was found when etiolated cabbage leaves from
well-headed plants were compared with leaves grown exposed to light (76).
Exposure of the etiolated pea (131) or cabbage plant (76) to light depressed
their aldehyde levels, and raised the acid auxin content in the cabbage. This
suggests that light is involved in the conversion of IAc to IAA, if the infer-
ence drawn from the preceding discussion is correct that IAA formation
proceeds at the expense of IAc.

With both living tissues and crude enzyme preparations of the pineapple
leaf (77), mung bean seedling (251), and pea epicotyl (136), tryptophan simul-
taneously raises the level of IAA and the ether-soluble neutral component
which was considered to be IAc. Assignment of identity was based on the
characteristics of neutral fractions obtained without tryptophan supplement.
The possibility that the neutral substances IAN or Et·IAA were likewise
formed after adding tryptophan cannot be definitely excluded. The argu-
ment that the nitrile or ester has not been shown to be present in the above
tissues or incubates is not conclusive, since the nitrile and ester were not
looked for. On the other hand, IAN is active in the *Avena* curvature test
(21, 251). When a known amount of IAN is incubated with coleoptile juice,
the sum of the resultant acid and neutral fraction's activities as assayed by
coleoptile curvature is appreciably less, and not more, than the original
nitrile activity (251). Yet, neutral fractions arising from tryptophan through
the action of pineapple leaf tissues or leaf preparations were found to have
low or negligible activities on direct assay. They yielded, however, an acid
fraction with considerable activity after treatment with soil, milk, or crude
Schardinger enzyme (77). Neutral fractions from mung bean seedlings
yielded similar results after conversion by coleoptile juice (251).

The above considerations would tend against the likelihood of concomitant
formation of IAN. They do not negate the possibility of Et·IAA formation.
The activity of Et·IAA in the *Avena* curvature test is in the order of 1/10
that of IAA (124). Hydrolysis of the ester would yield IAA. While one would
be loathe to postulate Et·IAA as an intermediate between tryptophan and
IAA, subsequent esterification of the acid may have occurred. However, it
is pertinent that infiltration of relatively high concentrations of IAA into
the pineapple leaf (75) or incubation of NAA with coleoptile juice (135) have
been reported to yield no significant increase of a neutral component with
auxin activity.

The keto-acid.—Studies on the conversion of IPyA to auxin tend to be

obscured by the spontaneous breakdown mentioned before of the keto-acid to both acid and neutral substances. Compensating for this breakdown, infiltration of IPyA into spinach leaf discs yields an increase of auxin activity (261). Increased levels of acid auxin, and a marked rise in the neutral component (IAc?) convertible to acid auxin by soil (131) were observed when pineapple leaf discs were similarly infiltrated (77). The experiments with crude enzyme preparations, however, are not particularly illuminating. Dialyzed cytoplasm of the spinach leaf decreases the auxin activity when incubated with IPyA at pH 6.8 (261), the level of acid auxin also being lowered with the pineapple enzyme at this pH (77). Yet at pH 5.0 the pineapple enzyme consistently caused an increased conversion of IPyA to acid auxin, but lower amounts of the neutral component assumed to be IAc were found at both pH (77). In more recent experiments with enzyme preparations from the mung bean seedling (251), again no conversion of IPyA to the neutral component (IAc) was observed. But the enzymatic formation of IAA from the keto-acid was clearly demonstrated, and found to have a pH optimum of 6.2 (251). The enzyme involved in this conversion was unaffected *in vivo* by doses of ionizing radiation 10^3 times greater than those causing inhibition of both native auxin formation and the formation of IAA from tryptophan (251).

Related to the conversion of IPyA, R. Moss in our laboratory has observed that the decarboxylation of the keto-acid is not appreciably activated by yeast carboxylase. It is therefore tempting to suggest that the apparent direct conversion of IPyA to IAA by mung bean and pineapple preparations was caused by endogenously generated H_2O_2 (127). It would be of interest to determine the effect upon IAA yields of adding H_2O_2 or of decreasing peroxide concentration by the addition of catalase or ethanol (28) to the enzyme-IPyA mixture.

The amine.—Like tryptophan, tryptamine gives a delayed curvature in the *Avena* test (202). The reaction is also delayed in the pea test (253). These responses could be attributed to low penetration rates, since the slight increase in IAc and IAA obtained on infiltration of tryptamine hydrochloride into pineapple leaf discs was considerably enhanced when the free base was used, or when the amine salt was incubated with the leaf enzyme (77). Corn embryo juice will also form IAA from tryptamine (268), and there are indications that the kidney bean plant may do so likewise (252). However, both the spinach leaf (261) and the mung bean seedling (251), or enzyme preparations from both plants, have been reported as unable to convert tryptamine to auxin. Cultures of *Ustilago zeae*, which readily produce IAA from tryptophan, can likewise not convert the amine (266). On this basis, the inference (241) of conversion to auxin as the explanation of plant response to certain analogous amines (114, 239) seems questionable.

On the other hand, plant amine oxidases which could produce the corresponding aldehyde have been characterized. Werle & Roewer (255) found a monoamine oxidase to occur in several plant species that would slowly

oxidatively deaminate tryptamine (cf. also 254). Kenten & Mann (117) intensively studied the amine oxidase of pea seedlings. It oxidizes tryptamine fairly readily, though not as rapidly as phenylethylamine or diamines. Indications of this enzyme's presence in extracts of lupin, lavender, and clover also were found, though interestingly enough, the enzyme appeared to be absent in extracts of spinach (cf. 261) as well as several other plants. Activity appears in pea seedlings, mainly in the cotyledons, several days after germination, and reaches a maximum in one to three weeks. Leaf extracts were more active than the stem, the root being lowest of the parts tested. The activity in adult plants was relatively low. It was shown that the primary attack on amines by the enzyme is an oxidative deamination yielding the corresponding aldehyde and H_2O_2. Of direct pertinence to the subject of auxin formation, Kenten & Mann (117) isolated phenylacetaldehyde as the dinitrophenylhydrazone from the reaction of the enzyme with phenylethylamine. The isolation of this aldehyde in about 40 per cent of theoretical yield (117) suggests a facile method for the laboratory synthesis of IAc using tryptamine and an amine oxidase. Moreover, the distribution and temporal change in activity found for this enzyme (117) is consistent with the pattern of distribution and temporal change in auxin production occurring during plant ontogeny (253). The correlation is not necessarily indicative of a causal relation, particularly in view of the apparent inability of certain plant species to utilize the amine in auxin formation.

The nitrile.—Bentley & Housley (22) found that negligible amounts of acid auxin were formed in IAN solutions containing *Avena* coleoptile sections. Considering the acid auxin in the ambient solution as IAA, the acid produced would indicate a less than one per cent conversion of the nitrile (113). Thus, only a very small part of the activity of the nitrile on coleoptile sections could be ascribed to production of the acid in the external solution (113), contrary to similar experiments with IAc (22). The reason for this discrepancy between IAc and IAN is not clear, and possibly indicates that hydrolysis of the nitrile is largely confined within the cells of the coleoptile (22). That hydrolysis of the nitrile to auxin does occur readily in the *Avena* coleoptile, and probably not in the pea stem, was clearly shown by Thimann (235). Coleoptile sections were placed in IAN solutions at concentrations inactive in the pea test. Auxin active in the pea test was found in both the ambient solution and in the pressed juice or ether extracts of the coleoptile sections. Computed as IAA, the amount of auxin found showed IAN conversions in the order of 25 to 50 per cent to have occurred. Furthermore, it was subsequently confirmed by chromatography that *Avena* coleoptiles convert the nitrile to IAA (217). There was no indication that indoleacetamide was produced in the process. The amide was suggested (22, 113) as a likely intermediate in a biological conversion of IAN to IAA, though it has but slight activity in the coleoptile section test (22).

The inactivity of IAN in several of the biological expressions of auxin action (21) where IAA is invariably active, was mentioned previously. Con-

sidering the experiments of Thimann (235) it appears probable that the positive expressions of IAN activity as an auxin were caused by *in vivo* conversion to IAA. It would be desirable to determine experimentally if the degree of auxin activity of the nitrile in various tissues could be correlated more generally with conversion ability. That some plant organs are apparently unable to accomplish this conversion, or do so weakly, raises doubt as to the participation of IAN as an intermediate in auxin biogenesis. There is insufficient information on IAN occurrence and mechanism of origin and hydrolysis to determine whether or not it does so participate.

Again the observed responses of plants to analogous nitriles are consistent with the interpretation of activity through hydrolysis to the corresponding active acids. Napthaleneacetonitrile (22), 2,4-dichlorophenoxyacetonitrile (22, 113), N-acetyl-indoleacetonitrile (214) and phenylacetonitrile (214) exhibit activity in the section test, but the naphthalene analogue, like IAN, is inactive in the pea test (239). Naphthaleneacetonitrile also gives a delayed response in the induction of tomato leaf epinasty (272), analogous to the delayed induction of parthenocarpy in the tomato noted for IAN (21). It may also be pointed out that the polar transport in the coleoptile of IAN (21), a neutral compound with low dissociation, can be more satisfactorily explained by assuming a preliminary *in vivo* conversion to IAA. Conversely, the inability of IAN to inhibit axillary bud development when applied to apical stumps of *Phaseolus* stems (21) suggests that the stem of this species, like that of the pea, is unable to hydrolyze effectively the nitrile. It may be conjectured that the greater activity of IAN when compared with IAA in the coleoptile section test (22, 23) is caused by more rapid intracellular penetration of the neutral lipophyllic nitrile. Synergistic interaction between IAN and IAA (167) either the endogenous acid or the acid derived from the nitrile, probably does not contribute to the greater activity of IAN in coleoptile sections (22).

The ester.—It is generally believed that esters exhibiting auxin activity do so by being hydrolyzed *in vivo* to the corresponding active acid. While this view is probably correct, it rests on no direct evidence. Kögl & Kostermans (124) found that the activity of *Avena* curvature of a homologous series of IAA esters decreased as the size of the alkyl chain increased. This activity pattern has been attributed to a parallel decrease in hydrolyzability (124, 253). Just as possible is a parallel impeded decrease in transport of the esters *per se* to reactive cells in the *Avena* coleoptile. For example, the isopropyl ester of IAA has an activity of 0.5 per cent of IAA in the *Avena* curvature test, 10 per cent in the *Avena* section test, and is equivalent in activity to IAA in the pea test (239). Perhaps most significant in pointing to hydrolysis before activity is the fact that there are no inactive acids known whose esters are active as auxins (120; cf. also 93, 124, 271).

General considerations.—It should be stressed that the capacity of a plant or plant preparation to convert a given compound to auxin or to an auxin precursor may be purely a facultative phenomenon. The existence of

such a conversion mechanism does not in itself constitute proof that the same process occurs in the normal biogenesis of auxin. To the plant physiologist concerned with auxins, a biochemical transformation is, or should be, little more than a provocative prototype that has no physiological meaning until experimentally integrated into the auxin or hormonal economy of the plant. Based on such precepts, and restricting consideration to those precursors known to occur in plants, the preceding discussion on auxin formation may be briefly summarized.

The evidence is convincing, though not conclusive, that tryptophan functions as a primary precursor of IAA in plants. It is the simplest explanation consistent with the data accumulated. Alternative explanations, however, cannot be disregraded. For example, little is known about the mechanism of tryptophan synthesis in the higher plant. The terminal stage of tryptophan biosynthesis may have a limited reversibility to a common precursor serving as the source of IAA. This reaction would not be incompatible with any of the observations here detailed. By any mechanism, it seems reasonable to ascribe the low yields of IAA obtained from tryptophan (and other precursors) using tissues or heterogeneous tissue preparations, to precursor demand by competing enzymes (265) as well as to IAA inactivation systems. Since scopoletin (6) and chlorogenic acid (83) at concentrations in the order of $10^{-5} 10^{-6}M$ effectively inhibit IAA-oxidase, the use of these compounds might minimize IAA destruction when crude enzyme preparations are employed.

IAc is transformed to IAA by the plant. A neutral compound with the characteristics of IAc is likewise formed from tryptophan. It is probable but not certain that the two are identical. The participation of the two frequently suggested intermediate products of tryptophan oxidation, IPyA and tryptamine, in the process of *in vivo* IAA formation has not been demonstrated. Although a mechanism exists in some plants for the conversion of the amine to IAc and IAA, other plants are unable to carry out this reaction. The same comment holds for the keto-acid. The widespread occurrence of IPyA and tryptamine in the plant has not been shown as yet, each having been identified only once. And it is an assumption as yet unvalidated that they arise by the orthodox oxidative deamination (28, 218) or decarboxylation (29, 57) of the amino acid when they do occur. Finally, both the origin of IAN and Et·IAA and the mechanism of their conversion to IAA is obscure, and evidence of their participation in auxin biogenesis has yet to appear.

It is, of course, possible that the intermediates between tryptophan and IAA are different in certain plants. A more tenable assumption to the reviewer is that a common, not yet clarified, pathway occurs for the primary genesis of IAA from the amino acid. In certain organs or stages in development IAA may be converted into mobile and possibly reserve precursors. As previously indicated, growing buds and leaves are active in auxin production, and are able to convert tryptophan readily to IAA. Even so, studies

with elongating shoots of the *Ginkgo* (85a) indicate that the young leaves and apical meristems do not transport auxin directly, but rather a precursor of auxin which is converted to the active form in the stem beneath (cf. also 98). The auxin produced on seed germination (44, 89) does not move acropetally in the shoot (253) or with basipetal polarity in the very young hypocotyl apex (108). Since an inactive precursor of auxin does move upward to the coleoptile tip (202, 245), the disappearance of auxin in the scutellum (56, 246) or first internode (89) [and perhaps the young hypocotyl apex of legumes (108)] may be attributed to conversion of the auxin to an inactive transport form (136). It should be pointed out, however, that the above "disappearance" may be literally destruction, and what is really transported is one of the same precursors involved in initial auxin synthesis. IAc might also be viewed (87) as an acropetal transport precursor if the molecular weight and pH sensitivity ascribed to this neutral precursor (186) are valid. A postulate of IAc (or even Et·IAA) as the transport form would have to reconcile the auxin activity of the aldehyde or ester in subapical coleoptile tissues, with the lack of direct activity shown by the precursor in the same tissues (186, 202). The suggestion of tryptophan or tryptamine as models for the transport precursor (253) still seems applicable, and is more compatible with the distribution of the tryptophan-converting enzymes in the coleoptile (260).

Conversely, if conversion of auxin to an inactive transport form does take place, the growth inhibitor obtained from the corn scutellum may be involved. Extractable by organic solvents or electrodialysis, where it moves to the cathode, it is an ultrafilterable molecule unaffected by acid or alkali (224). Though it acted as an inhibitor in the cress-root test, no growth promotion was observed at low concentrations (179, 224). Breis of *Avena* coleoptile tips converted the inhibitor to auxin (224). Moreover, the inhibitor could inactivate either pure IAA or endosperm auxin by apparently combining with the auxins (182, 224), the inactive product being reactivated upon acid or alkaline hydrolysis (224). Thus, both the inhibitor and inhibitor-auxin complex provide inactive models of a form which, if translocated, is potentially regeneratable to active auxin by the coleoptile tip. Their behavior in acid and alkali, and molecular size in the case of the complex, appear incompatible with the characteristics determined by Raadts for the mobile precursor. In her experiments (186), the more appropriate physiological technique of successive placement of agar blocks on coleoptile stumps was used to obtain the precursor. It is possible that the scutellum inhibitor functions as an inactivation mechanism in preventing the accumulation of auxin at the coleoptile base.

The concept of bound auxin as a storage reserve may also be mentioned. During the early stages of seed development, higher levels of auxin are found in the endosperm than in the embryo (14, 97, 146). The total content of extractable auxin rises, particularly as the embryo matures, and then falls as the seed ripens. Essentially, this pattern of change in auxin level has been noted for both monocot and dicot seeds (15, 97, 146, 148, 162, 163, 183). The

observed drop in auxin levels has been ascribed to conversion of the free aux-
in to a bound form (213) subsequently available to the embryo upon ger-
mination (89). That auxin becomes so bound in the seed, and that the bound
form functions as a reserve supply, have not been shown. The presence of
Et·IAA in mature seeds (190, 228) might merit consideration in this respect.

In view of the above discussion of auxin formation, it is perhaps redun-
dant to say that our understanding of the mechansims involved in auxin
biosynthesis and transformation needs clarification. The use of genetic mu-
tants, isotopically labelled compounds, and differential migration would
seem to be fruitful techniques in resolving the existing uncertainties.

DESTRUCTION

The discussion of auxin destruction is restricted to processes that decrease
auxin concentration, *in vitro* or *in vivo*, (*a*) by decomposition of the auxin
molecule to yield free inactive products; (*b*) by adsorption or complex forma-
tion ("binding").

Peroxides and acids.—Careful purification of diethyl ether before use as
an auxin solvent was long deemed necessary, since the organic peroxide pres-
ent in impure ether apparently inactivated auxin (253). Indeed, the reduc-
tion in biological activity on addition of H_2O_2 to auxin preparations has been
frequently used in the characterization of auxins and related substances
(e.g., 56, 89, 224, 246). It has been shown (198) that IAA is not actually in-
activated by either H_2O_2 or organic peroxides. Apparent destruction of the
auxin derives from interference by peroxides with both biological and colori-
metric assay methods. Thus, in the *Avena* curvature test the activity of IAA
exposed to H_2O_2 or ether-derived peroxide was restored by, respectively,
catalase treatment or diluting out the peroxide. Similarly, virtually complete
restoration of IAA as measured colorimetrically was obtained by first decom-
posing or extracting the peroxide. The presence of peroxide in auxin prepara-
tions could therefore lead to erroneous assay values unless the peroxide is
removed previous to assay. Quantitative data on auxin disappearance in mix-
tures which contain peroxide should be critically examined in view of the
above results.

Pure auxins exhibit different sensitivities to hot dilute mineral acids and
alkalis. IAA is inactivated by hot acid (122), and possibly even at a pH below
2 at room temperature (77). It is relatively (77, 100, 101, 131) stable in hot
dilute alkali (120). The reported sensitivity of IAc to both acid and alkali,
and the stability of IAN in dilute acid, was previously mentioned. The chem-
istry of these inactivations is not known, though the destruction by acid ap-
parently involves molecular oxygen. Little or no auxin destruction takes
place under inert atmospheres (92, 101, 104). Perhaps bearing on this ques-
tion are the results summarized in a preliminary report (105) on the nature
of the initial chromogenic reaction of IAA with Fe^{+3} in acid solution. It was
stated that oxidation occurs at the indole N with a one-electron shift to form
the N-N diindolyl derivative at maximum color intensity. The dimer is then
rapidly hydrolyzed to N-hydroxy IAA and IAA.

Radiation: visible.—Compounds with auxin activity known to occur in plants have no absorption bands in the visible spectrum when in inert transparent solvents. Hence, appreciable photolysis can be induced in this frequency range only in the presence of intermediary pigments or sensitizers able to absorb the light and convey the energy so absorbed to the auxin molecules. A number of compounds possess the ability to mediate the photoinactivation *in vitro* of both pure auxins and the auxin in plant extracts: eosin (33, 51, 53, 201); lycopin and carotene suspensions (125; but cf. 192); fluorescein (33, 51); thionine, rhodamine b, and methylene blue (33); aesculin and triphenyl-tetrazolium chloride (51); quinine sulfate (51, 53); scopoletin and methyl umbelliferone (6); lumichrome (58); and riboflavin (33, 34, 35, 43, 47, 51, 58, 59, 62, 83, 95, 192). Some of these compounds (viz., tetrazolium, quinine, aesculin) are colorless; they absorb light in the ultraviolet region and fluoresce in the visible (52). Their mediation of IAA photolysis may be attributed to the ultraviolet portion of the sunlight used for irradiation (51, 53). Accessory impurities found even in fractionated plant extracts can also apparently sensitize the photolysis of the auxin contained (110, 131, 145). Pure IAA in solution undergoes a slow light-accelerated decomposition (4, 76). In this instance the light receptor may be the traces of colored product arising through slow auto-oxidation of IAA. The implication (52) that the ability to sensitize the photoinactivation of auxin is necessarily related to the fluorescent property of the photoacceptor is somewhat misleading. The essential characteristic of the activated sensitizer is its ability to transfer energy, directly or indirectly, to the auxin molecule, the energy being of sufficient magnitude to enable auxin inactivation.

Riboflavin-sensitized photooxidation of IAA has been studied in detail, especially by Galston and co-workers (58, 59, 62), who discussed its implications for auxin-controlled phenomena such as phototropism and internode elongation (59, 62; cf. 60, 174, 195). IAA in solution is inactivated by white light in the presence of riboflavin, with the liberation of 1 mole CO_2 and absorption of slightly over 1 mole O_2 per mole IAA inactivated (58). This loss in CO_2, presumably from the carboxyl group of IAA, causes a rise in pH of the solution (33, 47). Brauner (33) used this pH change to estimate the amount of IAA photooxidized. The extent of IAA destruction under argon (58) was seen to be about 10 per cent of that in an aerobic control, with decoloration of the flavin taking place in the absence of air. If oxygen was later introduced into the anaerobic solution, IAA rapidly disappeared. It was concluded that activated riboflavin functioned as hydrogen carrier between IAA and oxygen. The empirical reaction mechanism was suggested (58):

$$Rbf + h\nu \rightarrow Rbf^* \qquad\qquad 1.$$

$$Rbf^* + IAA \rightarrow IAA(ox.) + Rbf \cdot H_2 \qquad\qquad 2.$$

$$Rbf \cdot H_2 + \tfrac{1}{2}O_2 \rightarrow Rbf + H_2O \qquad\qquad 3.$$
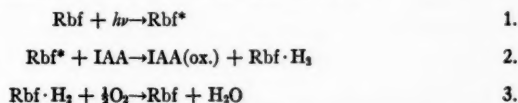
In a recent study, Brauner (35) substantiated the observation (58) that less riboflavin-sensitized inactivation of IAA occurs in the absence of air. The

addition of colloidal Pt, Mn^{+2}, or peroxidase had no effect on the rate of auxin inactivation in aerated solutions, indicating that H_2O_2 was not directly involved. Decreased IAA losses occurred in the presence of guaicol and ascorbic acid, and as ethanol concentration was increased. Inactivation efficiency rose under anaerobic conditions as the mole ratio of riboflavin to IAA was raised. Brauner (35) accordingly proposed that the IAA photolysis was accomplished by active oxygen derived from the water through a free radical mechanism. Equation 2 above was amended to:

$$Rbf^* + 2H_2O \rightarrow Rbf \cdot H_2 + 2OH \qquad \qquad 2a.$$

$$2OH \rightarrow H_2O + O \qquad \qquad 2b.$$

$$O + IAA \rightarrow IAA(ox.) \qquad \qquad 2c.$$

This formulation of the reaction sequence appears unjustified on the following grounds. First, most of the hydroxy-compound inhibitions observed could just as well be attributed to competition with IAA for activated riboflavin (reaction 2). Second, if photolysis of water did occur (reaction 2a), OH radical reduction by IAA (as well as other oxydizable solutes) would be anticipated, an oxidation whose occurrence is indicated in (79). Third, the above mechanism may also be examined on thermodynamic grounds, where it becomes analogous to one of the problems of photosynthesis. Since the O—H bond energy is approximately 110 kcal./mole (175), about 4.8 ev would be required for the dissociation of a single water bond. Thus, roughly 10 ev would be needed for reaction 2a, yet at 4400Å, the quantum energy (hc/λ) is about 2.8 ev. Unaccounted for is the mechansim whereby this energy requirement for water photolysis would be satisfied. It is likely that, as in most photoreactions, the intermediate formation of free radicals is involved. However, it is necessary to know the rate constants of reactions in which these radicals participate, as well as the intermediates and yields, in order to formulate specific reaction mechanisms. [Galston (62) calculated a quantum yield of 0.67 for IAA destroyed in the presence of riboflavin by a single irradiance at 4500Å. This yield can be considered only as an approximation. As he pointed out, it is probably less than the optimum, for no cognizance was taken of the positive effects of component concentrations, nor of temperature and pH (cf. 35, 58). Moreover, the disappearance of IAA with respect to radiation dose may proceed with or approach an exponential form (58). Using a single irradiance may thus introduce another source of error in a characterization of quantum efficiency.]

It was stated (59) that the product of IAA photolysis when sensitized by riboflavin was a condensation product of several cleaved indole rings. That the indole ring is to some extent lost in this oxidation was indicated by the decrease in Hopkins-Cole color both with IAA and other indolyl compounds (58). However, indolealdehyde is apparently likewise formed, having been identified in the reaction mixture after irradiation with white light by both chromatography and color reaction (47). In not strictly comparable experi-

ments with quinine sulfate (51, 52), a reaction product with the characteristics of skatole was also observed. In view of the stoichiometric liberation of CO_2 found with riboflavin (58), it seems likely that side-chain degradation of IAA occurs, possibly followed by ring activation on continued irradiation.

When biological tissues responsive to auxin are exposed to solutions of riboflavin (or other sensitizers) and auxin, the decreased growth response which takes place in the light undoubtedly involves auxin photolysis (34, 43, 51, 53, 62, 95). It is doubtiul whether endogenous photolysis of auxin is involved in the decreased growth of etiolated tissues exposed to low-light intensities. Galston & Hand (68) could find no differences in auxin content between darkened and illuminated pea tissues, but observed a decreased capacity of illuminated tissues to respond to auxin (cf. also 237). A similar decrease in sensitivity to auxin was found with pea tissues exposed to red light (65), and with cucumber hypocotyls exposed to red or blue light (106). Although white light increased the disappearance of IAA in solutions with etiolated pea sections (68), both illuminated and nonilluminated cucumber hypocotyls took up the same amount of auxin (106). However, pretreatment with red light decreased the removal of IAA from external solutions (65). Green stem tissues of the pea show an enhanced growth response to IAA under white light; here the limiting factor appears to be the photosynthetic elaboration of carbohydrate (64). The possible participation of auxin photolysis in tropisms has been reviewed elsewhere (60, 195, and by Brauner in the present volume). The mechansim of the light action in tropistic responses is still obscure, and possibly involves altered rates of auxin function or metabolism (60). The nonspecificity of sensitized photolyses of biological components, such as those that may be mediated by riboflavin itself (50, 58, 59, 61, 107, 152, 161), is not inconsistent with this view.

Pertinent to the nature of the pigment photoacceptor in phototropism are some recent observations of Reinert (192). He found that neither β-carotene nor carotenoid epoxides could mediate the *in vitro* photolysis of IAA. Carotene, however, reduced the riboflavin-sensitized photoinactivation of IAA, probably by competitive absorption of light. Similarly, *Phycomyces* grown on a medium which led to riboflavin but not carotene formation, was found to be phototropically more sensitive than when grown under conditions leading to production of both pigments. Accordingly, the high phototropic sensitivity to blue light exhibited by the coleoptile tip, which is higher in carotenoid content than the coleoptile base (40), was ascribed to the effect of this pigment in increasing the transverse gradient in intensity of the effective wave lengths. Such enhancement of lateral inequality of irradiance by "dead" absorption could apply, of course, to any of the proposed phototropic mechanisms, viz., transverse transport, photolysis, or biosynthesis of auxin. In this connection, mutant corn coleoptiles containing riboflavin but devoid of carotene were reported to show almost normal phototropic sensitivity to blue light (59, 60).

Ultraviolet.—The auxins have well defined absorption bands in the middle

ultraviolet spectrum. Hence, inactivation *in vitro* by direct photon absorption in this frequency range would be anticipated. Indirect inactivation by activated water might also be presumed at higher ultraviolet frequencies. Rapid inactivation of auxin solutions by ultraviolet irradiation had been early noted (130). Similar inactivations *in vitro* of indolepropionic acid (96), IAA (47, 48), and probably of 2,4-D.[2] (176) have been observed. With the last compound, some enhancement of activity in the pea test was also noted after irradiation (176), though the high concentrations used make the data difficult to interpret. Interestingly, *cis-trans* isomeric interconversions of cinnamic acid are accelerated by ultraviolet radiation. Thus, the active (253) *cis*-form was converted to the inactive (171) *trans*-isomer (26), while the reverse conversion has been likewise indicated (269). A similar isomeric interconversion probably occurs when the inactive *trans*-naphthalene-acrylic acid is irradiated by ultraviolet light (242). Irradiation of IAA in solution gives rise to indolealdehyde (47). At the same time a compound less acidic than IAA was formed, which was suggested to be a keto-acid intermediate in the formation of indolealdehyde (47). While it is clear that ultraviolet irradiation causes a lowered auxin level *in vivo* (41, 48, 149, 177, 184), it is again doubtful that direct photolysis of auxin is involved. In plant tissues, auxin is present at relatively low concentration in a heterogeneous milieu of ultraviolet absorbing chromophores. It seems more likely that the lowered auxin level found following ultraviolet irradiation is caused by altered rates of auxin metabolism. This interpretation is more clearly indicated in the analogous response of the plant exposed to ionizing radiation.

Ionizing.—An extensive study was made by Skoog (201) on the destruction of auxin by ionizing radiation. He found both auxin preparations from the plant and pure IAA to be highly radiosensitive toward x- and γ-radiation. Calculations subsequently derived from his data (78, 79) indicated ionic yields and inactivation kinetics suggestive of a radiation-initiated chain reaction. Apparent inactivation of auxin in the etiolated coleoptile and in green plants was observed following moderate doses of x-rays (201). Production of auxin in light-grown *Pisum* and *Vicia* plants was likewise inhibited by similar radiation dosages, although auxin formation in the *Avena* coleoptile appeared to be relatively insensitive to x-irradiation. The effects of x-radiation on auxin-regulated phenomena, such as shoot elongation and apical dominance, were correlated with the destruction of auxin and inhibited auxin production. Numerous morphological and morphogenic responses of plants to ionizing radiations have since been wholly or in part attributed to radiation-induced auxin destruction or depression of auxin levels: pollen germination (55), dwarfism (73), callus growth (88), flower formation (139), root growth (154), gall formation (248, 249), and general morphological changes of higher plants (85).

Recent (78, 79) and current studies indicate that pure IAA and IAA of plant origin exhibits no unusual sensitivity to high-energy radiation. The auxin disappears exponentially as a function of dosage, with initial ionic

yields in the order of unity. Protective action is readily afforded by the presence of cosolutes, cytoplasmic protein of the spinach leaf being particularly effective in this respect. Direct destruction of auxin in tissues unable to form auxin is negligible. For example, ionic yields of auxin inactivation in subapical sections of the *Avena* coleoptile are apparently in the order of 10^{-17} or less (75). Yet plants and plant tissues of the cocklebur, kidney and mung bean, and celery cabbage, do show a depressed free auxin level immediately following exposure to a low dose of x-rays, and a continued inhibition of auxin production (78; cf. also 201). At the same time the ability of the plant to utilize auxin in growth (201) or to destroy auxin through native inactivation mechanisms (75) is unaffected by low radiation doses. Auxin production was therefore suggested (75) as the reaction sensitive to radiation damage in a dynamic system of auxin formation and depletion. Inhibited auxin formation could thus entirely account for an *in vivo* depression of auxin levels via systems responsible for multimolecular turnover of auxin. It would avoid the necessity of postulating auxin photolysis in a biological medium exposed to several roentgens, where the ratio of potentially protective solutes (79) to auxin molecules is high. Substantiating the above explanation is the previously mentioned *in vivo* radiosensitivity and recovery pattern of the enzyme system converting tryptophan to IAA (251). The locus of radiosensitivity appears to be the enzyme-converting "IAc" to IAA. The neutral component with characteristics of the aldehyde piles up in the mung bean seedling following irradiation by low x-ray doses; moreover, enzyme preparations made from similarly irradiated plants show an inhibited ability to convert biological preparations of IAc to IAA (251).

The inactivation products formed when IAA in aqueous solution is irradiated are not known. A number of inactivation products would be anticipated, considering the photon energies of ionizing frequencies. The kinetics of IAA inactivation indicate that the products effectively compete with IAA for the oxidizing radicals derived from water (251). Spectral absorption curves obtained during irradiation suggest that inactivation at low O_2 tensions occurs chiefly by side-chain degradation (78). The moles of CO_2 released at various dosages, probably by decarboxylation, were consistently about 75 per cent of the moles of IAA inactivated. In the presence of air, inactivation appears to be caused chiefly by ring rupture. Though H_2O_2 is formed in irradiated IAA solutions, the peroxide is apparently not involved in IAA inactivation (81); moreover, the maximum concentration of peroxide formed was found (81) to be well below the level where spurious assay values for IAA result (198).

Binding.—The inability to secure complete recovery of IAA added to tissues or tissue preparations has been frequently noted (viz., 22, 29a, 133, 156, 157, 221). While some auxin undoubtedly disappears in tissues through utilization in the growth mechanism (32), there are indications that this system is quickly saturated by low absolute amounts of auxin (68), amounts which are less than those absorbed and inactivated (32, 68, 242). Destruction of the

auxin by IAA-oxidase action *in vivo* or *in vitro*, or possibly auxin photolysis *in vitro*, may be to a large extent responsible for auxin inactivation. However, sufficient information is available concerning other potentially operative in-activation mechanisms to render automatic assignment to the above causes unwarranted.

The juices of potatoes (156) and unripe cherries (157) contain a thermo-labile system that can apparently bind rather than destroy added IAA. The bound auxin can be released by incubation with pancreatin. Auxin can be released from many tissues or gross tissue components by incubation with more specifically proteolytic enzymes (cf. 74, 234). It is difficult to assess the validity of such auxin liberations. To some extent they are probably artifacts derived from autolytic conversion of tryptophan to auxin. However, there is more direct evidence that auxin protein complexes exist in plants. The as-sociation of auxin with isolated and purified proteins of the leaf (232, 259, 262) and seed (74) has been demonstrated. The release of auxin from these proteins by alkaline treatment may be justifiably questioned (194) in view of tryptophan conversion to auxin under alkaline conditions (82). It is doubtful, however, whether more than a fraction of the auxin released from the cyto-plasmic leaf protein by weak alkali arises from the amino acid (259), and there are indications that the auxin formed by alkali from seed proteins is unrelated to that released by proteolytic enzymes (74). Moreover, pure pro-teinases do liberate auxin from a number of the proteins isolated (74, 232, 259).

Some information concerning the nature of nondestructive binding of auxin is beginning to emerge. In part it gives foundation to the concept that nonspecific binding of auxin may occur in tissues, and throws light on the characteristics of auxin macromolecule association. Spontaneous binding of auxin to proteins would be anticipated as a protein-ion interaction. Smith and co-workers (207 to 210) studied the inhibition of *in vitro* activity of car-boxypeptidase by various carboxylic acids. Auxins were found to be effective inhibitors of the enzyme, one of the most active being IAA (210). There was no apparent correlation between activity of the compounds as auxins and their efficiency as inhibitors. Thus, as R-COOH compounds, in inhibition efficiency indole > phenyl > aliphatic > napthyl. *Cis-* and *trans*-cinnamic acids were ineffective, even at high concentrations. Both the R-residue and the carboxyl appeared to react with the enzyme, though the residue interac-tion was attributed to weak, nonspecific van der Waals' forces rather than primary chemical bonds (210). Somewhat closer to physiological applicabil-ity are the physical adsorption studies with 2-methyl-4-chlorophenoxy acetic acid with various adsorbents by the monolayer technique (37). The auxin bound strongly to long-chain amines, weakly to ketones, and did not react with phenols, alcohols, and sterols. It reacted with the seed prolamine gliadin, but not with lecithin at pH >4, a pH above which the auxin binds as an an-ion. No interaction was seen with the model lipoprotein gliadin-lecithin, indi-cating that a lipid may block formation of the protein-ion complex. Although

cress and tomato plants are more sensitive to methyl-chlorophenoxyacetic acid than is wheat, monolayers of the wheat coleoptile gave a fivefold greater adsorption than those made from cress and tomato terminal meristems. The suggestion was made (37) that species sensitivity and reactivity may be markedly influenced by the extent of auxin adsorption to sites not concerned in the physiological response (cf. 242). It would be desirable to determine if species correlation such as the above hold more widely, particularly when more comparable organs are used. The concept of auxin binding on physiologically "inactive" sites in the intact organism is likewise of interest in that it is capable of being extended in interpretation of enhanced auxin responses in the presence of hemi-auxins (187, 188), and structural analogues of auxins (206, 236), i.e., "sparing" action, as well as the synergisms between auxins themselves (cf. 241). Indeed, data on the retardation of root growth by auxin and auxin-antagonists lend themselves readily to mathematical analysis as pure adsorption phenomena (112).

There are indications that the binding of auxin to protein may occur by different mechanisms. The addition of IAA *in vitro* to protein obtained from the pea root yielded a complex from which the auxin could readily be removed by acetone or by acid (200). This complex may be considered analogous to typical protein-anion interactions where the binding is chiefly through electrostatic and van der Waals' forces (119). Even so, the energy with which IAA thus binds to proteins appears to be relatively high. This is indicated by the efficiency with which IAA inhibits carboxypeptidase (210). In this connection, the interaction of IAA with crystallin serum albumin as a model system was evaluated by the dialysis equilibrium technique (225). A binding of three moles IAA per mole protein was indicated, with a first binding energy of ca. −6 kcal./mole. This free binding energy for IAA is appreciably greater than those derived for analogous phenyl and phenoxy acids with albumin in a similar system, and is of the same magnitude as the corrected binding energies calculated for dinitrophenolate and picrate (226).

On the other hand, auxin could not be eluted from the auxin-protein complexes isolated from the spinach leaf by exhaustive extraction with ether or a mixture of ether-ethanol (262). Similarly, lack of auxin elution by ether was noted with the protein-complexes of the wheat grain, although the auxin could apparently be removed from the wheat proteins by electrodialysis (74). Prolonged electrodialysis, acidified-acetone extraction, and dialysis against acid or alkali appeared unable to remove the auxin from the cytoplasmic complex of the spinach leaf (259). Nor were acetone and dilute acid or alkali effective with the IAA-protein complex of the pea root (200). The above results indicate a strong attachment of the auxin to the protein in native complexes.

Parenthetically, a point may be made in relation to the binding energies involved in the complexing of auxin *in vivo*. The free energies of auxin-complex formation in coleoptile tissues were calculated (151) using parameters derived from the growth response of the coleoptile. Such values, with precise

implications, may be somewhat misleading when based on the response of a chemically heterogeneous organ segment. It is entirely possible, and indeed likely (32, 37, 68, 242), that nonspecific binding of auxin occurs in tissues, perhaps proportionately to the auxin supplied. Equilibrium constants obtained by kinetic treatment of growth versus concentration of different auxins (or antiauxins) appear to be useful in evaluating the relation of growth-substance structure to relative biological activity (151). The extension of these constants to derive the free energies involved in formation of the "active" complex in tissues appears questionable.

An endergonic coupling of auxin to protein that may be considered as a mechanism whereby strong auxin-complexes could be formed in the plant has recently been described (200). Excised pea root tips were able to bind IAA *in vivo* to nonparticulate protein. Protein-bound IAA was detected colorimetrically when external IAA concentrations of 10^{-6} M were used, increasing amounts being bound up to an IAA concentration of 10^{-3} M. It should be pointed out that these IAA concentrations are much greater than concentrations which may be termed physiological for the root (231). NAA inhibited the binding of IAA (200), which suggests that auxins other than IAA may react. Endogenous protein-binding was also inhibited by dinitrophenol, iodoacetate, cyanide, and azide. The effect of these inhibitors led to the demonstration that protein binding *in vitro* could be almost doubled by ATP[2] when root preparations depleted of endogenous ATP were used. The auxin-protein complex so formed was cleaved by coenzyme A *in vitro* to yield the protein and an IAA derivative which, though water soluble, was insoluble in ether at pH 3. It seems plausible that native IAA in the plant may be in part protein-bound by energy-coupled reactions sparked by ATP. Whether such protein-binding of auxin has any direct significance in the growth reaction remains to be determined.

The CoA effect just mentioned may be related to another CoA-auxin reaction concomitantly reported (138). The addition of various auxins, including IAA, to a mixture of tomato-ovary mitochondria, ATP, and CoA, caused a disappearance of free sulfhydryl groups as measured by absorbance changes with the nitroprusside test. All four components were required for the reaction. The —SH disappearance, presumably in CoA, was proportional to the concentration of auxin from about 10^{-11} to 10^{-8} or 10^{-7} M, higher concentrations of auxin inhibiting the reaction. The magnitude of the sulfhydryl effect could be directly correlated with the known auxin activity of the various compounds examined. Aside from their pertinence to concepts of auxin action mechanisms, these findings suggest that one form of bound auxin may be the thiol-ester of CoA. The —SH groups could be completely regenerated by making the medium alkaline. Regeneration presumably liberated the bound auxin, and suggested (138) that this process might be involved in the release of auxin observed after treatment of tissues with mild alkali (cf. 234). It also seems probable that the thiol-ester was the IAA derivative found on treating

the protein complex of the pea root with CoA (200). It should be pointed out, however, that the actual coupling of auxin to CoA was not demonstrated in either of the investigations (138, 200). Moreover, in view of the effectiveness of the low concentrations of auxin employed in the latter work (138), it is possible that the auxin action in mediating —SH diasppearance could have been in part indirect, and not solely through thiol-binding of the auxin.

IAA-oxidase.—The enzyme system which accelerates the oxidative destruction of IAA was first studied in detail by Tang & Bonner (220, 221). Subsequent work on this enzyme to 1951 was discussed in a preceding review (136). Since then, several studies on the IAA-oxidase of the pea (5, 6, 63, 67, 70, 72) and pineapple plant (83) have appeared. The enzyme is probably widely distributed (221) particularly in etiolated shoots and roots (221, 247). IAA destruction by the enzyme in the green plant is ordinarily not appreciable (221, 247). This lack of activity is probably caused by the high content in green tissues of a soluble inhibitor of the enzyme (221), since inactive preparations obtained from light-grown pea plants were activated by repeated acetone washes (63).

Indolealdehyde was suggested as the reaction product of IAA oxidized by the oxidase system (247). The R.Q. of 1 for the reaction (83, 220, 247), the ether solubility and neutral character of the product (220, 247), and the ability to secure an insoluble dinitrophenylhydrazone from the reaction mixture (247), may be considered as presumptive evidence for the aldehyde (cf. also 103). Since pure indolealdehyde is available, and may be chromatographed as such on paper (46, 47, 54, 217), or as the dinitrophenylhydrazone on silicic acid columns (76), the formation of the aldehyde by IAA-oxidase action should be readily verifiable.

Galston *et al.* (67) has presented additional evidence indicating that the IAA-oxidase system of peas consists of a light-activatable flavoprotein (62) coupled through H_2O_2 to a peroxidase. The system was inhibited by catalase (67, 70), the inhibition being reversed by blue light but not by red or green (67). Metallic ions (63), Mn^{++} in particular (63, 67), also inhibited the activity. Their inhibitory effects were likewise reversed by blue light (63). The action of these ions was attributed to presumably competing H_2O_2 decomposition (67). The activity of the IAA-oxidase was increased by the addition of H_2O_2 in the dark (67, 70), and by the addition of crystallin peroxidase in the light (67). These findings indicated that the H_2O_2 concentration is rate-limiting in the dark, and that H_2O_2 production is increased by light to the extent where the peroxidase becomes limiting. It is likely that the major destruction of IAA by the system is peroxidative, since crystalline horseradish root peroxidase readily oxidized IAA in the presence of H_2O_2 (67). The IAA-oxidase system was fractionated into a flavin-containing component, and a peroxidase which, in the presence of H_2O_2, was active toward IAA and conventional peroxidase substrates. The activity of the peroxidase fraction toward IAA was augmented by addition of the flavin fraction. Moreover,

xanthine oxidase added to crystalline peroxidase was able to simulate the IAA-oxidase activity. In this instance, however, a substrate other than IAA was required for the generation of H_2O_2 (67).

The activation by light of xanthine oxidase (29), which was used as support for a flavoprotein activation by light (63, 67), was recently reinvestigated (199). No evidence of xanthine oxidase photoactivation could be obtained (199). This does not rule out, however, the possibility of another flavin system acting as a photoreceptor.

Andreae (5) found that scopoletin was a competitive inhibitor of IAA destruction by the oxidase. The stimulation of scopoletin oxidation by IAA was shown (6) to support the concept (63, 67) that IAA gives rise to peroxide on oxidation. However, H_2O_2 did not stimulate IAA oxidation in either the light or dark (6). It was concluded (6) that IAA served as the source of the peroxide needed for its own oxidation, and that H_2O_2 will only become rate-limiting when other substrates which compete for the peroxide are present. This interpretation may explain the acceleration of IAA-oxidation by H_2O_2 noted with crude juice (70), but seems inconsistent with the positive effects of added H_2O_2 secured with purified oxidase preparations (67).

Methyl umbelliferone was found to accelerate the destruction of IAA (but not of scopoletin) by the oxidase in both the light and dark (6). Maleic hydrazide and 2,4-D were also found to enhance the oxidase action on IAA in the light and dark. Evidence was presented that the above three stimulators of IAA destruction acted via acceleration of the light-activated stage (6). This stage ordinarily limits the rate of IAA oxidation (6, 62, 67, 83). Methyl umbelliferone also inhibited the growth of cress roots in the dark (6), indicating that growth inhibitions previously noted for this coumarin derivative are not necessarily related to a photosensitized inactivation (94).

The enhancement of IAA activity by 2,4-D had been reported previously (66, 69, 70). Since 2,4-D inhibited catalase but not the peroxidase activity (70), it was suggested that the enhancement of IAA oxidation might have been caused by increased availability of peroxide. This explanation was questioned (6), since it was shown that H_2O_2 was not rate-limiting for IAA oxidation, that 2,4-D probably did not increase peroxide production or utilization by peroxidase, and that concentrations of catalase much higher than existed in preparations of the oxidase would be required to exert a significant competition for H_2O_2. [See also Table II in (72).] Moreover, it was recently shown (72) that the apparent enhancements with 2,4-D previously noted were undoubtedly caused by the phenols contaminating even some recrystallized preparations of 2,4-D. Halogenated phenols, particularly analogues of 2,4-Dichlorophenol, were found to be powerful accelerators of the IAA-oxidase activity (72). The mechanism whereby phenols enhance the oxidase appears uncertain. Several possibilities were considered by Goldacre, *et al.* (72): participation of the phenol in an oxidation-reduction system, reversal by the phenol of a naturally occurring inhibitor of the oxidase, and function of the phenols as an enzymatic cofactor.

The destruction of IAA in solution by pea stem sections also was en-

hanced by dichlorophenol, being accompanied by a decreased growth of the sections (72). Furthermore, halogenated phenols were observed to inhibit effectively the activity of catalase (71). Several other heme proteins (peroxidase, cytochrome oxidase, hemoglobin), were unaffected by dichlorophenol except at high concentrations (71). The inhibition of catalase by dichlorophenol does not explain the phenol effect on the IAA oxidase, however, since preparations of the oxidase free of catalase activity were still capable of being activated by dichlorophenol (72). It seems unlikely that catalase normally inhibits the action of IAA-oxidase *in vivo;* catalase was found to be present only in the particulate fractions of pea epicotyl breis, whereas the oxidase activity was restricted to the "soluble" fraction (72).

Preparations of the pineapple plant were found by Gortner & Kent (83) to contain a very active IAA-oxidase system as well as a strong inhibitor of the enzyme. The inhibitor was considered to have characteristics of a polyphenol compound. As with the pea enzyme (6, 63), the effect of the inhibitor could be reduced by dialysis or exposure to light. A number of marked differences were found, however, for the pineapple enzyme. The enzyme appeared to be distributed throughout the plant, with highest activity in etiolated shoot tissues, and lowest activity in the green leaves and roots (cf. 221, 247). In contrast to the optimum of pH 6 to 7 determined for the oxidases of the pea (220) and bean root (247), the optimum for the pineapple enzyme was about pH 3.5. The enzyme was virtually inactive above pH 6. It requires Mn^{++}, whether dialyzed or undialyzed, while evidence for the Mn^{++} requirement of the enzyme of the pea (63, 67, 72, 247) or bean (247) is conflicting. Although cyanide and so-called copper reagents inhibit the IAA-oxidase regardless of source (83, 220, 247), the strong copper-chelating compound, 8-hydroxy-quinoline, either had no effect, or enhanced the activity of the pineapple enzyme (83; cf. 247). In contrast to the stimulation of the pea enzyme noted for maleic hydrazide (6) and dichlorophenol (72), these compounds had little or no effect on the pineapple enzyme.

The IAA-oxidase from the pea shoot, bean root, and pineapple plant could be essentially the same enzyme system. If so, an explanation of the discrepancies discussed above is not as yet readily apparent. Conversely, it could be concluded (83) that the IAA-destroying enzymes so far studied are all distinct systems. The various findings do not appear inconsistent with the views, first, that the major portion of the IAA destruction by the enzymes is accomplished by oxidation via a peroxidase-H_2O_2 action. [Indeed, peroxidase preparations alone were reported to be able to carry on a limited oxidation of IAA (67), probably via endogenously generated peroxide. An accelerated oxidation of IAA was also reported (116) for peroxidase to which Mn^{++} had been added (contra 67)]. Second, that an inhibitor of the enzyme which is deactivated on irradiation with visible light occurs in various preparations of the oxidase. Third, that the action spectrum of the light effect and characteristics of the photoreceptor are strongly suggestive of a flavo-protein or flavin-containing system.

The common response of the different preparations to light are in some

respects difficult to reconcile with the observed characteristics of the enzyme in the intact plant. Thus, light-grown pea seedlings have a high content of the inhibitor, and a low IAA-oxidase activity (63, 221). Illumination of the etiolated pea seedling results in a progressive increase in the content of inhibitor and a decrease in oxidase activity (221). Speculations have been made as to possible roles of the IAA-oxidase in phototropism (67), in the prevention of auxin accumulation to supra-physiological levels (27, 69, 221, 247), and in interaction of the enzyme with endogenous inhibitors as a morphogenetic determinant (6, 83). No physiological role for this enzyme has been demonstrated experimentally as yet.

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